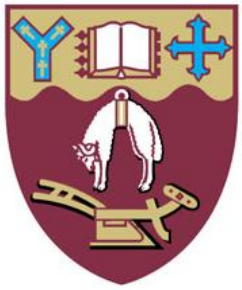
Cytotoxicity of Hypochlorite-oxidised Proteins



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ABBREVIATIONS

78NP 7,8-dihydroneopterin

7KC..... 7-ketocholesterol

AAPH..... 2,2-azobis(2-amidinopropane) dihydrochloride

ACN..... acetonitrile

ApoB-100..... apolipoprotein B-100

ANOVA..... analysis of variance

AOPP……advanced oxidation protein product

BHT ..... butylated hydroxytoluene

BSA..... bovine serum albumin

CD36..... cluster of differentiation 36 scavenger receptor

CO_2..... carbon dioxide

$CuCl_2$..... copper chloride

Cu-oxLDL……copper-oxidised LDL

DHE ..... dihydroethidium

DMSO..... dimethyl sulphoxide

DNA..... deoxyribonucleic acid

DOPA..... 3,4-dihydroxyphenylalanine

EC ..... endothelial cells

EDTA..... ethylenediaminetetraacetic acid

FBS ..... foetal bovine serum

GSH ..... glutathione

H_2O ..... water

HDL ..... high-density lipoprotein

HMDM..... human monocyte derived macrophages

HOCl..... hypochlorous acid

HOCl-oxLDL……hypochlorite oxidised low-density lipoprotein

HPLC ..... high performance liquid chromatography

KBr..... potassium bromide

KOH..... potassium bromide

LD_{50} ..... median lethal dose

LH ..... polyunsaturated fatty acyl group

LDL..... low-density lipoprotein

$LOO^{\bullet}$ ..... lipid peroxyl radical

LOOH ..... lipid hydroperoxide

MBB..... monobromobimane

MDA ..... malondialdehyde

mLDL..... minimally oxidised low density lipoprotein

MTT ..... 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide

NaCl ..... sodium chloride

NaOCl ..... sodium hypochlorite

NaOH ..... sodium hydroxide

NADPH..... reduced nicotinamide adenine dinucleotide phosphate

NO..... nitric oxide

$NO_2^{\bullet}$ ..... nitrogen dioxide

$ONOO^-$ ..... peroxynitrite radical

O_2 ..... molecular oxygen

$OH^{\bullet}$ ..... hydroxyl radical

OxALB……..oxidised albumin

OxLDL..... oxidised low density lipoprotein

P- ..... protein

PBS ..... phosphate buffered saline

PMA..... phorbol-12 myristate 13-acetate

PUFA ..... polyunsaturated fatty acid

REM..... relative electrophoretic mobility

$RO^{\bullet}$ ..... alkoxyl radical

$RO_2^{\bullet}$..... peroxyl radical

ROS..... reactive oxygen species

Rpm..... revolutions/minute

RPMI-1640 ..... Roswell Park Memorial Institute cell medium

SEM ..... standard error of the mean

SDS ..... sodium dodecyl sulfate

SMC ..... smooth muscle cell

SR-A ..... scavenger receptor type A

TBA ..... 2-thiobarbituric acid

TBARS..... thiobarbituric acid reactive substances

TCA ..... trichloroacetic acid

VLDL..... very low density lipoprotein

$^{v}/_{v}$..... volume/volume

$R^{\bullet}$ ..... free radical

$^{w}/_{v}$ ..... weight/volume

ABSTRACT

The role of cell death in atherosclerosis remains ill-defined, however, a growing body of evidence suggests that cell death stimulates atherogenesis through the induction of inflammation and enlargement of the necrotic core. Although there is solid evidence to suggest that lipid oxidation and toxicity are linked, indications that protein oxidation may play an important role in cytotoxicity are numerous. The abundance of dead cells in atherosclerotic plaques and their co-localization with HOCl-modified proteins provides an opening for the suggestion that the products of protein oxidation may be at the heart of oxLDL-induced cell death. Examination of the modification of LDL and albumin by HOCl, and the cytotoxicity of these oxidised molecules were the focus of this study, along with the elucidation of their cell death mechanisms toward U937 cells.

Measurement of lipid peroxidation markers, TBARS and 7-ketocholesterol, showed no significant increase in HOCl-oxLDL compared to native levels although all α-tocopherol had been lost. In contrast there was a large loss of tyrosine, of which a small percentage went to dityrosine, indicating that the protein moiety of LDL was the main target of HOCl attack. Albumin became fragmented and smeared on SDS-PAGE gels with increasing HOCl/BSA molar ratios. In addition there was significant reduction in tyrosine levels and a small increase in dityrosine.

Both HOCl-oxLDL and oxidised albumin (oxALB) caused concentration-dependent cell viability loss in U937 cells following a significant drop in intracellular GSH concentration, coinciding with a peak in oxidative stress. Removal of chloramines with methionine significantly reduced the toxicity of oxALB, but at higher concentrations this effect was reduced. This was in contrast to HOCl-oxLDL where the removal of chloramines had no effect on its toxicity. Morphological observations of cell swelling, cell membrane integrity loss and rupture, along with flow cytometry results indicate that U937 cells underwent necrosis, but only after intracellular GSH was lost. Intracellular GSH and cell viability loss were prevented by 200 μM extracellular 7,8-dihydroneopterin (78NP), indicating that 78NP scavenging of ROS generated in

response to the oxidised proteins was sufficient to prevent cell death. This study demonstrates the cytotoxicity of HOCl-damaged LDL and albumin is likely due to a common oxidative product or structural motif which may be active within atherosclerotic plaques.

1. INTRODUCTION

1.1 Overview

Atherosclerosis is a condition of the major arteries in which progressive occlusion of the vessel occurs and is the result of an on-going inflammatory process (Lusis, 2000). The involvement of low density lipoproteins (LDL) in the pathogenesis of atherosclerosis is widely accepted. According to the oxidative modification hypothesis of atherogenesis, LDL within the artery wall undergoes oxidative modification, forming oxidised LDL (oxLDL) which is then taken up by macrophage cells in an unregulated manner, triggering their transformation into lipid laden foam cells (Libby *et al.*, 2002).

Oxidised lipids are thought to be involved in the cell death process that characterises that progression of the fatty streak to advanced atherosclerotic plaque. Although cytotoxicity of lipids to many cells types has been reported (Clare *et al.*, 1995, Larsson *et al.*, 2006, Lizard *et al.*, 1998), such studies have failed to acknowledge the role oxidised proteins are likely to play in cellular death. Myeloperoxidase, a heme protein secreted by activated neutrophils, plays a critical role in the killing of evading micro-organisms. However, MPO can also induce damage to surrounding tissue (though the over production of potent oxidant hypochlorite), and is thought to contribute to the pathogenesis of several diseases including atherosclerosis (Malle *et al.*, 2000; Daugherty *et al.*, 1994; Baldus *et al*., 2002). Further to this, hypochlorite (HOCl)-modified proteins that have been identified in human atherosclerotic plaque, and HOCl has been shown to transform LDL to a high-uptake form without significant lipid oxidation (Hazell *et al.*, 1996). Accumulation of reactive protein oxidation products such as advanced oxidation protein products (AOPPs) may also cause further damage to other cellular macromolecules in the vicinity. A clear understanding of the products of HOCl-induced oxidation, together with analysis of oxidation products *in vivo* will allow for evaluation of the role in atherosclerosis of myeloperoxidase, and the phagocytic cells that produce it.

1.2 Atherosclerosis

Atherosclerosis is a progressive disease, characterised by the accumulation of lipids and fibrous elements in the large arteries (Libby, 2002). It primarily presents as cardiovascular disease, cardiac infarction and stroke. Overall it is responsible for approximately 50% of deaths in the westernized world (Lusis, 2000). Atherosclerosis manifests itself through progressive morphological changes, from the precursor 'fatty streaks' to advanced fibrous lesions. Early lesions of atherosclerosis consist of accumulations of cholesterol-laden macrophages termed 'foam cells'. These fatty streaks although clinically insignificant, are precursors of more advanced lesions, characterised by accumulation of lipid-rich necrotic debris and smooth muscle cells (Lusis, 2000). These advanced lesions (or plaques) can become increasingly complex with calcium and iron deposits, often with an unstable necrotic core. Although advanced lesions may grow sufficiently large enough to slow, or block blood flow, it is more likely that the danger of these plaques comes from clinical complications. The plaque can rupture or erode, resulting in complete occlusion of the blood vessel, consequently leading to myocardial infarction or ischemic stroke.

1.3 Oxidised LDL and atherosclerosis

Epidemiological, clinical and genetic studies have provided clues for the underlying causes of atherosclerosis. It is known that individuals whose levels of low density lipoprotein (LDL) are elevated by lifestyle or genetic heritage show an increased risk for myocardial infarction (Heinecke, 1998). Although this correlation has been made, LDL does not appear to display atherogenic properties *in vitro*. Macrophages incubated with high levels of LDL failed to internalize excess cholesterol (Goldstein *et. al.*, 1979; Fogelman *et. a.l*, 1980), yet cholesterol-laden macrophages are a hallmark of early atherosclerotic lesions, and LDL is the major cholesterol transporter in blood. One hypothesis to account for this anomaly and a driving factor for many studies is the 'oxidative modification' hypothesis. The implication is that LDL must be somehow altered or modified prior to its uptake by macrophages and then taken up, not by the native LDL receptor, which is tightly regulated, but rather by some alternative macrophage receptor.

The oxidative modification hypothesis of atherogenesis is based upon four potentially atherogenic effects of oxLDL. These effects are: chemotactic activity that facilitates the recruitment of circulating monocytes to the endothelium; the inhibition of macrophage migration from within the artery back to the plasma compartment; enhanced uptake of LDL by macrophages via scavenger receptors contributing to the formation of foam cells; and cytotoxicity resulting in endothelial integrity (Steinberg *et al.*, 1989).

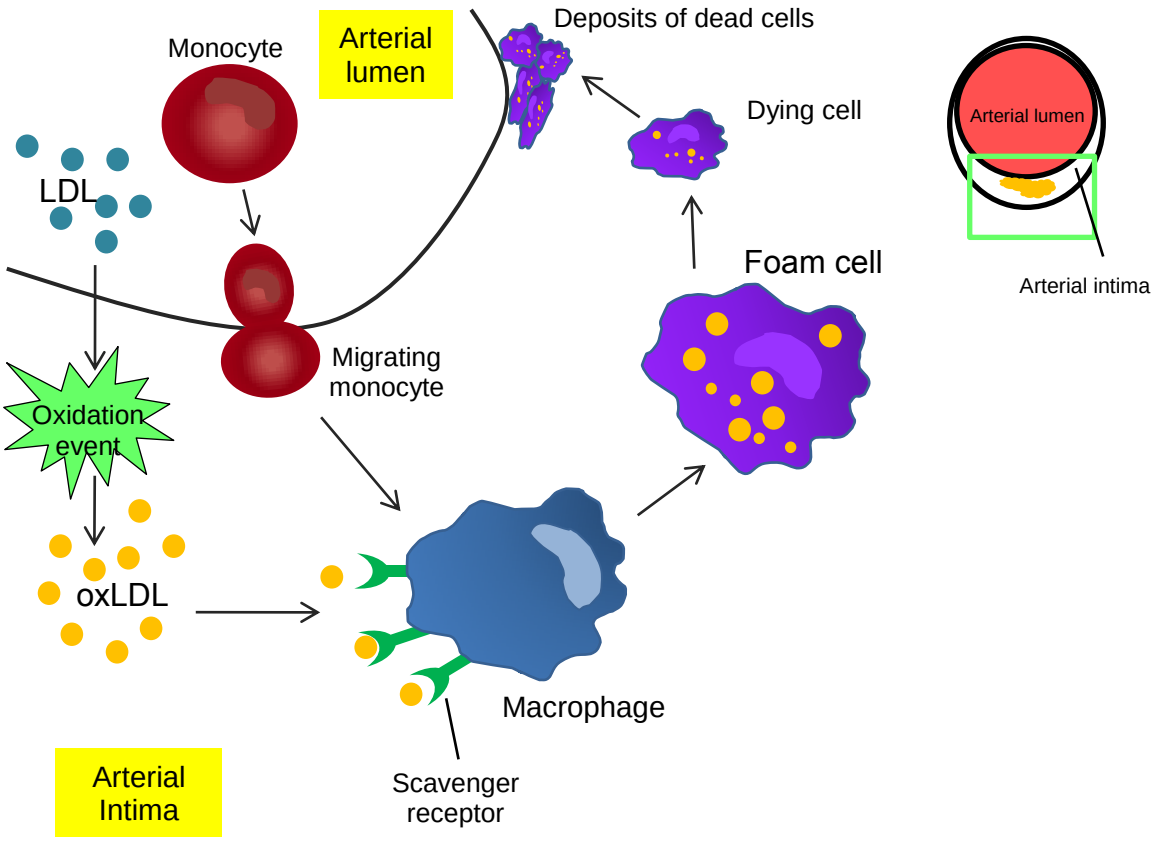


Figure 1.1 Oxidative modification hypothesis and its causal role in atherogenesis. Cells enter the artery wall as monocytes and collect in the intimal layer, before differentiating into larger macrophage cells. Native LDL does not promote foam cell formation because the activity of the LDL receptor is tightly regulated, however upon oxidative modification of the LDL molecule, scavenger receptors on the macrophage recognize and bind oxLDL internalizing in an uncontrolled high uptake manner, resulting in so called foam cells. These cells no longer have the same physiological properties as a macrophage and aggregate within the intima. OxLDL is thought to attract and recruit cells of various types including monocyte and smooth muscle cells to the fatty streak site leading to formation of the atheroma and subsequent plaque.

Lesion initiation is thought to occur with the accumulation of LDL and lipoproteins in the sub-endothelial matrix. Trapped LDL is then thought to undergo modifications such as oxidation, proteolysis and aggregation, which may be caused in part by exposure to oxidants released by vascular cells (Lusis, 2000).

OxLDL and other forms of modified LDL are taken up by macrophages via scavenger receptors, such as CD36 and SR-A (Steinberg *et al.*, 1989). Foam cells are loaded with droplets rich in cholesteryl esters, derived mainly from arterial wall macrophages, originating from circulating monocytes that have penetrated into the sub endothelial arterial intima (Steinberg, 2000), formed when macrophages internalize oxLDL in an uncontrolled manner. They are the main constituent of the early stage atherosclerotic lesion, known as the fatty streak (figure 1.1).

Fatty streaks evolve into complicated atheromas though the replication and migration of smooth muscle cells (SMCs); these accumulate and lay down an abundant extracellular matrix in response to cytokines, growth factors and reactive species secreted by activated macrophages and T lymphocytes (Lusis, 2000). SMC migration and proliferation signifies an important acceleration in the process of atherosclerosis as it significantly expands the lesion size (Berliner & Heinecke, 1995). Cell death appears to be central to the formation of a necrotic core in advanced atherosclerotic plaques (Gieseg *et al.*, 2009). Together with the wealth of experimental evidence that confirms the cytotoxicity of oxLDL (Baird *et al.*, 2004, Bjorkerud and Bjorkerud, 1996, Harada-Shiba *et al.*, 1998, Reid and Mitchinson, 1993) and its presence within atherosclerotic plaques (Brown *et al.*, 1997, Fu *et al.*, 1998, Ylaherttuala *et al.*, 1989), it is likely that oxLDL is a major driver of cell death *in vivo* and hence, necrotic core development.

Advanced atherosclerotic lesions consist of a fibrous cap of smooth muscle cells and dense connective tissue covering surrounding areas with necrotic debris, inflammatory infiltrates, macrophage foam cells, cholesterol clefts, calcium deposits and secreted extracellular matrix proteins (Crisby, 1997; Glass and Witztum, 2001). Plaque rupture with subsequent thrombus formation is considered the most common cause of acute ischemic infarction, but the mechanisms responsible remain poorly understood. The formation of a lipid core appears to be associated with progressive tissue destruction and eroding the surrounding fibrous cap (Newby, 1998). This degeneration is likely to

be of key importance for plaque rupture. Macrophages within the intima have been shown to secrete cytokines that amplify a local inflammatory response and matrix metalloproteinases that can degrade the fibrous cap, weakening the plaque (Halliwell & Gutteridge, 2007). Cells in the ruptured fibrous cap and the overlying thrombus have exhibited signs of DNA fragmentation, thus indicating that cell death in the cap had occurred close to the time of rupture (Newby, 1998).

OxLDL is known to stimulate macrophages to induce foam cell formation and inflammatory responses. Although the pathological aspects of oxLDL have been well studied, the formation, distribution, and overall fate of oxLDL *in vivo* remain unclear.

1.4 Cell death and oxidative stress

OxLDL has been implicated as a key indicator in a number of plaque promoting processes. OxLDL has been shown to be taken up by macrophages in a rapid and uncontrolled manner leading to formation of cholesterol filled foam cells, the major component of fatty streaks. However, oxLDL may also modulate atherogenesis by inducing death of cells and tissues within the inflammatory site (Ermak *et.al*, 2008).

Two types of cell death, termed necrosis and apoptosis have been extensively described. Necrosis is characterised by cellular swelling, rupture of plasma membrane, and leakage of cellular components during cell lysis. Apoptosis is characterised by DNA fragmentation, alterations of nucleus morphology, and cell fragmentation (Salvayre *et.al.*, 2002).

Apoptosis is the mechanism which removes cells that have been produced in excess, or that have been damaged, through activation of an internally encoded suicide program. The cell becomes fragmented and enclosed in membrane bound apoptotic vesicles, which are removed by neighbouring cells though phagocytosis. Distinctions between apoptosis and oncosis (primary necrosis), can be drawn by distinct morphological changes in the cells which are characteristic to each cell death mechanism. Cells can be identified as going through apoptosis by condensation of the nucleus, cytoskeletal disruption, cell shrinkage, and membrane blebbing (Crisby *et.al*, 1997). Necrosis is

characterized by a gain in cell volume, swelling of organelles, rupture of the plasma membrane and subsequent loss of intracellular contents (Martinet *et.al*., 2011).

Cell death induced by oxLDL is well demonstrated in cultured cells *in vitro* including U937, THP-1 cell lines, smooth muscle cells (SMC) and endothelial cells (EC) and human monocyte derived macrophages (HMDM). Cell death also occurs in atherosclerotic areas, potentially leading to defects in the endothelial cell lining, to necrotic core formation, and possibly plaque rupture or erosion (Salvayre, 2000). Although both oxLDL and apoptotic/necrotic cells are present in atherosclerotic lesions, a direct link between oxLDL and apoptosis remains to be demonstrated *in vivo*.

Various stimuli in the plaque including high levels of oxidative stress, depletion of cellular ATP, impaired clearance of apoptotic cells and increased intracellular calcium may cause necrotic death (Martinet *et.al*., 2011). Although the role of necrosis in atherosclerosis remains ill-defined, a growing body of evidence suggests that necrotic death stimulates atherogenesis through induction of inflammation and enlargement of the necrotic core. In addition, necrosis contributes to plaque instability by releasing tissue factor, matrix degrading proteases and pro-angiogenic compounds (Ermak *et.al*., 2008; Halliwell & Gutteridge, 2007; Martinet *et.al*., 2011; Newby, 1998)

The type of oxidative modification may play a role in the pro-apoptotic effects of oxLDL. Two separate caspase-dependent apoptotic pathways have been implicated in oxLDL induced apoptosis (Ermak *et.al*., 2008); the extrinsic pathway – mediated by death receptors, and downstream activation of caspase-8/caspase-3; and the intrinsic mitochondrial apoptotic pathway – involving cytochrome c and caspase-3/caspase-9.

Studies have shown the involvement of reactive oxygen species (ROS) in apoptosis, induced by different agents including oxLDL (Assinger *et.al.,* 2010). Indeed, lipid peroxidation, production of ROS and down-regulation of antioxidant defence have been observed in several apoptotic situations (Assinger *et.al*., 2010; Ermak *et.al*., 2008). The intracellular sources contributing to ROS generation in monocytes include cyclooxygenases, lipoxygenases, mitochondrial respiration and predominantly NADPH oxidase. Oxidative stress and inflammation represent central and causally linked features of the atherosclerotic process. Integral to the oxidative modification hypothesis

is that ROS can oxidise lipid or protein (apolipoprotein B-100) components of LDL, hence transforming LDL to a high uptake form that is internalised by macrophages and resulting in foam cell formation. (Patel *et.al*., 2000).

There are a number of sources of ROS that contribute to the progression of atherosclerosis. Myeloperoxidase enzyme (MPO) is responsible for HOCl production (Glass and Witztum, 2001), and both MPO enzyme and HOCl (detected as 3-chlorotyrosine) have been found co-localising in atherosclerotic plaques (Daugherty *et al*., 1994; Malle *et al*., 2000; Sugiyama *et al*., 2001). ROS can contribute to lesion formation in many ways. Depending on the types and availability, oxLDL taken up by macrophages may eventually impose an oxidative stress on these cells causing death, which is thought to contribute to necrotic core formation. The presence of oxidation products of lipids and (lipo)proteins in atherosclerotic plaques further support the roles ROS play in the lesion development (Stocker & Keaney, 2004; Leeuwenburgh *et al.*, 1997).

1.5 Low density lipoprotein

1.5.1 Native LDL

LDL is the main carrier of cholesterol within the body and is crucial to the controlled transport and metabolism of lipids in the bloodstream. LDL is a spherical molecule consisting of approximately 20% protein made up entirely of a single protein, apolipoprotein B-100. This is embedded in a monolayer surface of polar phospholipids and cholesterol, surrounding a core of neutral cholesterol esters and triglycerides (Esterbauer et al., 1992). LDL is clearly abundant in lipids, of which almost half are fatty acids. Of the fatty acids, approximately half are polyunsaturated fatty acids (PUFAs), depending on the donor, which makes LDL highly susceptible to free radical-mediated oxidation. The PUFAs in LDL are normally protected against free radical attack and oxidation in the plasma by a range of antioxidants, including α-tocopherol, ascorbate, and urate.

LDL uptake by cells occurs either via a receptor-mediated pathway of the LDL-receptor or by non-specific endocytosis. LDL interacts with the LDL-receptor via ionic interactions between clusters of amino acids and the acidic amino acids of the receptor (Brown & Goldstein, 1979). Modifications to the LDL molecule can cause conformational changes to the apolipoprotein B-100 (apoB-100), which affects the binding of modified LDL to the LDL receptor (Esterbauer *et al.*, 1992). This results in high, unregulated uptake of modified LDL via scavenger receptor-meditated endocytosis, in which the cell rapidly internalizes cholesterol leading to foam cell formation (Esterbauer *et al.*, 1992).

The macrophage recognizes these oxidized lipids through a family of pattern recognition receptors known as the scavenger receptors. Lesional macrophages express at least six classes (A-G) of structurally unrelated scavenger receptors that allow the unregulated uptake of oxidized or acetylated LDL (oxLDL, AcLDL) (Moore & Freeman, 2006) with the majority of oxidised LDL taken up through a class B receptor, CD36 (Pluddemann *et.al.*, 2007). In addition to recognition and internalization of modified lipoproteins, scavenger receptors perform additional functions that can modulate atherosclerotic lesion progression, including (1) induction of macrophage apoptosis, (2) clearance of apoptotic cells and debris, and (3) activation of cellular signalling pathways regulating lipid metabolism and inflammation (Moore & Freeman, 2006). Thus, these multi-functional receptors regulate numerous pathways that affect both the initiation and progression of atherosclerosis.

In contrast to the single long chain polypeptides of apoB-100 in native LDL, oxLDL has many proteolytic fragments derived from apoB-100. The fragmented protein is thought to have been covalently modified by lipid peroxidation products such as aldehydes. Compared to native LDL, oxLDL contains depleted levels of PUFAs and antioxidants, massively increased levels of lipid peroxides and their degradation products, increased amounts of oxidised cholesterol products and enzyme break-down products and various protein oxidation species (Parthasarathy *et al.*, 1999).

1.5.2 LDL oxidative modifications

It appears from the literature that the most plausible and biologically relevant modification of LDL is oxidation. LDL can be oxidatively modified by all major cells of the arterial wall. Different types of oxLDL can be produced depending on the oxidation conditions. Of influence are the type and concentration of oxidant, level of antioxidants and modification of apoB-100. There is potentially a continuous spectrum of degrees of oxidation and a great deal of molecular heterogeneity in what we call "oxidized LDL".

In the early phase, mild oxidation of LDL results in the formation of minimally modified LDL (mLDL) in the sub-endothelial space. MLDL is very different in composition from heavily oxidised LDL (see figure 1.2). Cholesterol is still the prominent sterol, apoB-100 still binds to the LDL receptor, and incubation of mLDL with macrophages does not form foam cells (Itabe *et al.* 2011). However, a significant proportion of unsaturated cholesterol esters and phospholipids have been oxidised to hydroperoxides and short-chain aldehydes. It is not cytotoxic, which is most likely due to the retention of significant levels of α-tocopherol (Gieseg *et.al*, 2009).

It is known that LDL must be modified sufficiently to promote its uptake by macrophages. The oxidation necessary for this is quite significant with up to 50% of the cholesterol converted into 7-ketocholesterol and other oxysterols (Itabe *et.al.*, 2011). Furthermore, most of the unsaturated fatty acids are oxidised to a complex mixture of products and apoB-100 is extensively fragmented, derivatised and cross-linked. Due to the extensive modification of the molecule and apoB-100 protein, oxLDL is no longer recognised by the LDL receptor but is taken up avidly by the scavenger receptor pathway in macrophages, leading to excessive cholesterol ester accumulation and foam cell formation.

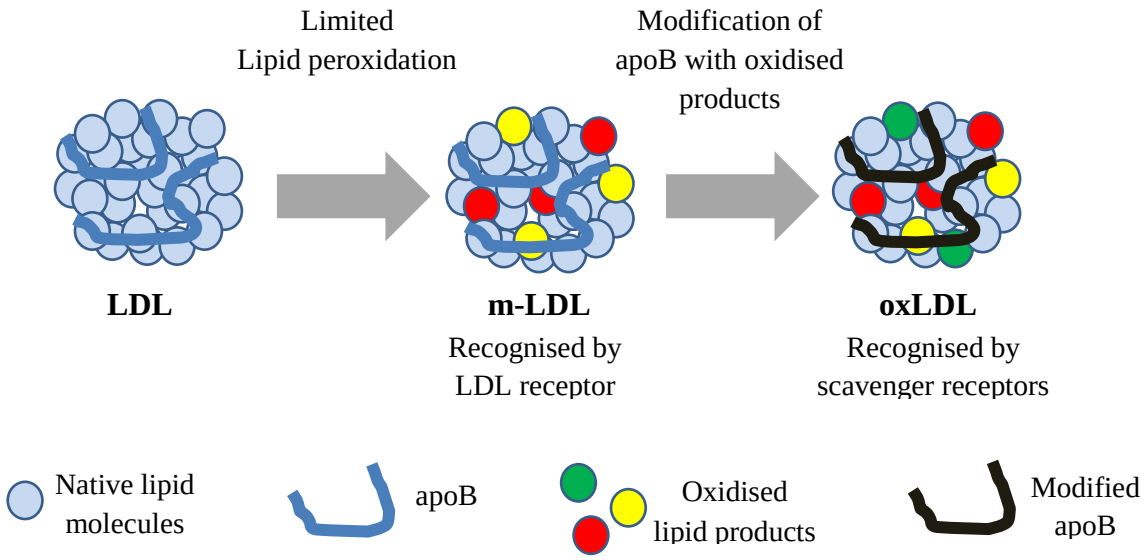


Figure 1.2: Hypothetical progression of LDL to oxLDL. LDL is thought to be modified in a stepwise manner during the generation of oxLDL. In the initial phase of modification, the lipid components react with oxidants, resulting in radical chain reactions that produce many types of lipid oxidation products. Subsequently lipid oxidation products react with the apoB protein to generate adducts and cross-links. Radicals can attack the apoB protein directly, resulting in oxidative changes of amino acid side chains and the cleavage of peptide bonds. M-LDL may contain lipid oxidation products without extensive protein modification, because it binds to LDL receptor rather than scavenger receptors. As modification on the apoB protein proceeds, its relative electrophoretic mobility changes greatly, as it loses the affinity to LDL receptor, and, in turn, becomes a ligand of scavenger receptors. Adapted from Itabe *el.al.*,2011)

OxLDL contains a number of oxidised protein products that may be of importance to its mechanism of cytotoxicity. The destruction of tryptophan residues on apoB-100 is an early and potentially important event in the copper-mediated oxidation of LDL (Esterbauer *et al.*, 1995) and it has been shown that LDL oxidation mediated by the macrophage myeloperoxidase product, hypochlorous acid, preferentially modifies the protein component of lipoproteins (Gerry *et al.*, 2008). In addition, it has been shown that protein hydroperoxides are generated at relatively high concentrations on apoB-100 during copper-, AAPH-generated peroxyl radical- and cell-mediated LDL oxidation (Gieseg *et al.*, 2003). The oxidation of the LDL protein moiety causes the loss of structural integrity of the apoB-100 protein, but also results in the loss of select amino acids, carbonyl formation, protein fragmentation, and aggregation via protein cross-linking (Esterbauer *et.al.*, 1990; Fong *et.al.*,1987).

A number of different types of LDL modifications are known to be recognised by these scavenger receptors, including aggregation, oxidation, chemical alteration (e.g., acetylation) and glycation (Pluddemann *et.al.*, 2007). Each of these changes is likely to induce multiple effects and that there is likely to be considerable cross-over between the different modifications. Although it is unclear which particular species are responsible for oxidative modification of LDL *in vivo*, many studies have shown that a range of oxidation mechanisms which exhibit some of the same biophysical properties characteristic of atherosclerosis.

Metal ions

Most of the cells present in the arterial intima can promote LDL oxidation, but it arguably requires the presence of transition metals, micro-concentrations of iron or copper in the culture medium; and it has been shown that and metal chelators can block cell-mediated LDL oxidation (Yoshida *et.al.*, 2010). Even in the absence of cells, high concentrations of free metal ions can oxidise LDL (Heinecke, 1998).

The most widely used procedure for preparing oxLDL for use in model systems involves incubation for 12 or more hours in either cell culture media or phosphate buffered saline supplemented with Cu^{2+} ions in the range of 5 to 100 µM. The copper ion induces lipid peroxidation, and subsequently, the chemical modification of the apoB-100 protein side chains with reactive lipid peroxidation products, such as 4-hydroxynonenal (4-HNE), and malondialdehyde (MDA). The presence of adducts of these oxidized products on apoB-100 protein has also been immunologically confirmed in atherosclerotic lesions (Esterbauer *et al.*, 1993).

The oxidation of LDL occurs via a lipid peroxidation chain reaction that is driven by the formation of lipid peroxyl radicals. The oxidation is typically described in terms of its three consecutive time phases; the lag phase, propagation phase and decomposition phase. LDL oxidation with free metal ions begins when a C-H bond in a polyunsaturated fatty acyl group (LH) is attacked by a free radical ($R^{\bullet}$), forming a lipid alkyl radical *(reaction i)* (Esterbauer *et al.*, 1990). Once formed, the carbon-centered PUFA radical reacts quickly with molecular oxygen to create a lipid peroxyl radical *(reaction ii).* The newly formed lipid peroxyl radical then reacts with a hydrogen atom

of an adjacent PUFA, forming a lipid hydroperoxide and a new PUFA radical *(reaction iii)* (Esterbauer *et al.*, 1993), which again yields a lipid peroxyl radical that will react with another PUFA molecule creating a chain reaction.

$$LH + R^{\bullet} \longrightarrow L^{\bullet} + RH \qquad (i)$$

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet} \qquad (ii)$$

$$LOO^{\bullet} + LH \longrightarrow LOOH + L^{\bullet} \qquad (iii)$$

$$LOO^{\bullet} + AH \longrightarrow LOOH + A^{\bullet} \longrightarrow A \qquad (iv)$$

During the lag phase, antioxidants within LDL initially compete with these chain propagating reactions by scavenging lipid peroxyl radicals *(reaction iv)* so that minimal lipid peroxidation occurs (Esterbauer *et al.*, 1993). Once the LDL has become depleted of endogenous antioxidants, the propagation phase commences and the PUFAs within LDL are thought to be rapidly oxidised to lipid hydroperoxides at a rate that increases exponentially. Decomposition of lipid hydroperoxides follows in the next phase, to yield a wide range of products including aldehydes, hydrocarbon gases, epoxides and alcohols. The lipid alkoxyl radicals and peroxyl radicals cause secondary oxidative damage to cholesterol, generating a range of oxysterols. The breakdown of lipid hydroperoxides to peroxyl and alkoxyl radicals generates isoprostanes and short chain aldehydes, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA). These lipid-derived aldehydes bind lysine residues of amino acid side chains of apoB-100 (Gerry *et al.*, 2008).

Enzyme systems

Substantial evidence supports the notion that oxidative processes contribute to the pathogenesis of atherosclerosis and coronary heart disease. Studies in cell culture have identified a number of enzyme systems that could in principle play a role in the oxidation of LDL. These include NADPH oxidase, 15-lipoxygenase, myeloperoxidase, the mitochondrial electron transport system, and others (Malle *et.al.*, 2006). Which of these contribute to LDL oxidation *in vivo* and to what extent is still uncertain, but over the last ten years, considerable data has been obtained in support of the hypothesis that

oxidants generated by the heme enzyme myeloperoxidase play a key role in oxidation reactions in the artery wall.

Macrophages secrete myeloperoxidase, which amplifies the oxidative potential of hydrogen peroxide by generating cytotoxic oxidants (Sugiyama *et.al.*, 2001). Myeloperoxidase is the only known human enzyme to generate hypochlorous acid, a highly reactive species (Heinecke, 1998). Specific oxidation products of hypochlorous acid such as 3-chlorotyrosine, α-chloro fatty aldehydes, and hypochlorite-modified proteins have been found in human atherosclerotic lesions (Westendorf *et.al.*, 2005). Moreover, an increasing number of studies demonstrated that LDL oxidized by hypochlorite (HOCl-oxLDL) *in vitro* is able to mimic fundamental atherogenic processes including, formation of foam cells (Hazell *et.al.*, 1993), activation of phagocytes with increased intra- and extracellular formation of reactive oxygen species (Kopprasch *et.al.*, 1998), and adhesion to endothelial cells (Kopprasch *et.al.*, 2004).

Oxidation markers confirm that HOCl attacks preferentially the LDL-protein moiety, inducing oxidative damage of apoB-100. Moreover, HOCl oxidation increases electrophoretic mobility of LDL. This increase in REM is thought to be due to modification of the apoB-100 lysine residues, with a concomitant increase in the net negative charge of the LDL particle (Ermak *et.al*, 2008).

Myeloperoxidase, which is present and catalytically active in human atherosclerotic lesions, promotes LDL oxidation *in vitro* by a variety of mechanisms that do not require metal ions; however, the exact mechanism by which it oxidises LDL is uncertain. One pathway for damage may be the tyrosyl radical, which promotes dityrosine formation and peroxidation of lipids. The detection of elevated levels of dityrosine in LDL isolated from lesions implicates the tyrosyl radical in oxidative damage. Elevated levels of 3-chlorotyrosine in lesion LDL provides even stronger evidence for the operation of the myeloperoxidase pathway *in vivo* because 3-cholorotyrosine is a specific marker for oxidative damage by MPO (Heinecke, 1998). In striking contrast, there appears to be little evidence that free metal ions, the most widely studied *in vitro* LDL oxidation system, plays an important role in the early disease process. However, the detection of elevated levels of *o*- tyrosine and other markers for metal-catalysed LDL oxidation in advanced atherosclerotic lesions supports the hypothesis that metal ions, perhaps

released by cellular breakdown, might contribute to the development of advanced lesions (Malle *et.al.*, 2006).

1.6 Hypochlorous acid

Hypochlorous acid (HOCl) is a powerful 2-electron oxidizing agent and a weak acid with pKa approximately 7.5, hence it is present in approximately equal mixture with its conjugate base hypochlorite (-OCl) at pH 7.4. For the purpose of simplicity, HOCl/-OCl will be referred to as HOCl in this thesis. It has important antibacterial properties, but excessive or mis-directed production of HOCl has been implicated in several diseases, including atherosclerosis, arthritis and some cancers associated with inflammation (Klebanoff, 2005).

1.6.1 Sources of HOCl

Stimulated neutrophils and monocytes, cells present under inflammatory conditions, produce HOCl via the MPO-catalyzed reaction of H_2O_2 with Cl^- *(reaction v)*. HOCl is a highly reactive oxidant and is thought to play an important role in both microbial killing and inflammatory tissue injury by neutrophils. HOCl reacts with a wide range of biological target molecules, including antioxidants, lipids and proteins to form chloramines, which are in turn, also powerful oxidizing agents (Zabe et.al 1999).

$$H_2O_2 + Cl^- + H^+ \xrightarrow{MPO} HOCl + H_2O \qquad (v)$$

Myeloperoxidase (MPO)

Myeloperoxidase (MPO) is a hemeprotein that is abundantly expressed in polymorphonuclear leukocytes (neutrophils) and secreted during their activation. The presence of a peroxidase in the cytoplasmic granules of leukocytes was suggested at the beginning of 20th century but it was the early 1940s that it was purified for the first time. Native MPO is a covalently bound tetrameric complex of two glycosylated alpha

chains (MW 59 – 64 kDa) and two unglycosylated beta chains (MW 14 kDa) (Podrez *et al*., 2000). MPO is sequestered in granules of neutrophils, where it comprises 2 to 5% of total neutrophil proteins (Halliwell and Gutteridge, 2007). It is also present in monocytes and in certain subtypes of macrophages and has been found up regulated in various disease states (Green *et al.*, 2004; Nagra *et al.*, 1997). Active MPO can lead to the formation of a range of oxidation products by catalysing the oxidation of a range of substrates and by the reaction of the oxidation products with other substrates. MPO uses H_2O_2 to oxidise halide ions such as chloride, bromide and iodide to form hypochlorous, hypobromous and hypoiodous acid, respectively (Harrison & Schultz, 1976; Halliwell & Gutteridge, 2007). MPO can also oxidise thiocyanate anions into hypothiocyanite (Hampton *et al.*, 1998). However, the high chloride ion concentrations in phagocyte cytoplasm and extracellular fluids suggest that HOCl is the major physiological product of MPO activity (Thomas & Fishman, 1986). Indeed, 28 to 70% of the H_2O_2 produced by neutrophils has been detected as HOCl (Foote *et al*., 1983; Weiss *et al.*, 1982).

MPO appears critical for oxidative killing of microorganisms in experimental systems. Neutrophils isolated from the blood of MPO-deficient individuals were inefficient in killing a variety of microorganisms (Lehrer & Cline, 1969; Kitahara *et al*., 1981). In addition, inhibitors of MPO such as azide, cyanide and salicylhydroxamic acid impair killing by normal cells (Klebanoff, 1970; Humphreys *et al.*, 1989).

Following phagocytosis, neutrophils undergo degranulation and MPO is rapidly released into the phagosome. However, leakage or secretion of MPO to the outside of the cell through incomplete closure of the phagosome or "frustrated phagocytosis" also occurs (Vissers *et al.*, 1985). This leakage of MPO can induce damage to surrounding tissue, and is thought to contribute to the pathogenesis of several diseases (Malle *et al.*, 2000; Daugherty *et al.*, 1994; Baldus *et al*., 2002).

1.6.1 Biological targets of HOCl

HOCl is a powerful oxidising agent that can react with many biological molecules such as DNA, lipids, proteins and free thiols. How vulnerable a particular target is towards oxidation by HOCl depends on: what else is present to compete with HOCl, relative concentrations of HOCl and other biomolecules, and on the molecules accessibility to HOCl (Folkes *et al.*, 1995; Pullar *et al.*, 1999). HOCl can react with $O_2^{\bullet-}$, amines and H_2O_2 to generate $OH^{\bullet}$, chloramines and singlet oxygen, respectively (Klebanoff, 2005). MPO also uses H_2O_2 to oxidize a variety of aromatic compounds, tyrosine residues (Klebanoff, 2005) and nitrite (Eiserich *et al.*, 2002) to generate their respective radicals.

Reactions of HOCl with membrane constituents resulting in loss of cellular integrity could be involved in bacterial killing as well as cytotoxicity/tissue injury caused by these inflammatory cells. They could also promote atherosclerosis by contributing to oxidative modification of LDL particles either through direct interaction or indirectly after transfer of HOC1-modified lipids from other sites (inflamed tissue, erythrocytes) to LDL (van den Berg *et.al.*, 1993). Chlorination of unsaturated fatty acids and cholesterol to chlorohydrin derivatives has been demonstrated (Pullar *et.al.*, 1999), which suggests that the lipid component of cell membranes could be susceptible to attack by HOCl. However, this reaction is relatively slow with chlorohydrins only being detected in cells after exposure to cytotoxic HOCl concentrations (Carr *et al.*, 1996; Vissers *et al.*, 1998). HOC1-modified lipids can exert cytotoxic and biophysical effects. HOCl-modified cholesterol oxidation products have diverse cytotoxic effects. Epoxy-, keto-, and hydroxy-derivatives of cholesterol affect de novo sterol biosynthesis, DNA synthesis, cellular functions, and cellular growth and proliferation (van den Berg *et.al.*, 1993).

Thiol groups and thioethers such as methionine are most readily oxidised at a rate approximately 100 times that of amine groups (Pullar *et.al.*, 1999). HOCl can also halogenate cell constituents; the most favoured chlorinating reaction is with amines to form chloramines (Halliwell & Gutteridge, 2007). Chloramines while longer-lived and less reactive than HOCl retain two oxidising equivalents, in which they utilize to oxidise thiols and heme proteins thus extending the reactivity of HOCl. Chloramines are

also known to be toxic to cells and bacteria, dependent on structure and their ability to cross the plasma membrane (Tatsumi & Fliss, 1994; Thomas, 1979) Additionally, chloramines can break down to reactive aldehydes which themselves are cytotoxic (Hazen *et.al.*, 1996).

HOCl also reacts with a range of low molecular weight biomolecules and antioxidants. HOCl reacts with GSH and ascorbate (Folkes *et al.*, 1995). In addition to high reactivity with free thiols such as GSH, HOCl can also oxidise protein thiols, though their reactivity may vary based on their accessibility and pKa (Pullar *et al.*, 1999).

HOCl has also been shown to cause rapid necrotic cell death, characterized by a rapid loss of cellular ATP and cell lysis without caspase-3 activation in HMDM cells (Yang *et.al.*, 2011). The mechanism by which HOCl is toxic to cells is proposed though destabilization of cytosolic calcium control resulting in the failure of both the mitochondria and lysosomes (Yang *et.al.*, 2011).

1.6.2 Effects of HOCl-oxLDL

Although the existence of HOCl-oxLDL has been known as a probable oxidative modification of LDL for many decades, it has not been utilized as much as some of the other experimental models. Our knowledge of how HOCl-oxLDL reacts *in vivo* and the role it plays in atherosclerosis is limited and not without holes.

Internalization of modified LDL into macrophages is a key component to the oxidative modification hypothesis. Not only is HOCl-oxLDL known to be internalized *via* classB scavenger receptors CD36 and SR-BI on the macrophages (Assinger *et.al.*, 2010; Ermak *et.al.*, 2008; Westendorf *et.al.*, 2005), but it has been shown that it up regulates CD36 and PPARγ levels in a dose- and time-dependent manner (Westendorf *et.al.*, 2005). Blood platelets play a central role in advanced atherosclerotic blood vessels, where an ulcer or fissure in the fibrous cap of the atheroma serves as an agonist that transforms the platelet into a major pro-thrombotic offender. HOCl-oxLDL stimulates the platelet Ca^{2+}-ATPase and leads to a decreased intracellular Ca^{2+}. Others have also noted

differences in the properties of Cu^{2+}-oxidised versus HOCl-modified lipoproteins (Zabe *et.al.*, 1999). The addition of HOCl-oxLDL to human platelets has not only shown its ability to stimulate their aggregation (Volf *et.al.*, 2000) but also strongly induces formation of ROS, and the impairment of this process by apocynin (Assinger *et.al.*, 2010) implies an important role of NADPH oxidase.

HOCl-oxLDL is known to contribute to a specific and directed migration of inflammatory cells, by induction of chemokine synthesis in human monocytes (Woenckhaus *et.al.*, 1998) and neutrophils (Terkeltaub *et.al.*1994). HOCl-oxLDL also exerts stimulatory effects on various human polymorphonuclear leukocytes (PMNLs)*;* including respiratory burst, degranulation, enhanced production of reactive oxygen metabolites, enzyme secretion (Kopprasch *et.al.*, 1998), and adhesion to endothelial cells (Lehr *et.al.*, 1995b; Kume *et.al.*, 1992). HOCl-oxLDL induced PMNLs adhesion to endothelium involves the generation and action of ROS and can be completely prevented *in vivo* by the antioxidant vitamin C (Lehr *et.al.*, 1995a). This, together with the observation that only HOCl-oxLDL stimulated PMNLs showed a significant respiratory burst with an extracellular release of both superoxide anions and hydrogen peroxide (Kopprasch, *et.al*, 1998), suggest that formation of ROS may indeed constitute a key event in leukocyte-endothelial cell interaction. Alternatively, HOCl-oxLDL can increase the adhesive properties of both leukocytes (Lehr *et.al.*, 1995b), and endothelial cells (Kume *et.al.*, 1992) by enhancing the expression of adhesion molecules on these cells (Kopprasch *et.al.*, 1998).

Cell death stimulates atherogenesis through induction of inflammation and enlargement of the necrotic core as well as contributing to plaque instability (see section 1.4). It is important to note that HOCl-oxLDL displays cytotoxic properties and should be acknowledged for the role it plays in atherosclerosis. HOCl-oxLDL induces apoptosis in all arterial cells, including monocytes, U937 cells (Ermak *et.al.*, 2010) and THP-1 cells (Vicca *et.al.*, 2000; Vicca *et.al.*, 2003). The nature of the apoptotic program varies depending on the oxidation type of LDL. The mechanism by which HOCl-oxLDL induces apoptosis was recently elucidated by Ermak *et.al.*, (2010) who showed apoptosis was induced by HOCl-oxLDL in U937 cells via the caspase-dependent pathway leading to activation of the caspase-3. The latter is activated to a minor extent

by direct cleavage by caspase-8, and to a major extent indirectly by mitochondrial involvement and subsequent activation of caspase-9 (Ermak *et.al*, 2010).

1.7 Protein oxidation

The formation of reactive oxygen groups upon protein is considered to be a significant contributor to cellular damage and as an indicator of the interruption to normal biological systems. For a long time, membrane lipids were thought to be the main cellular targets for oxidative damage due to their proximity and tendency to undergo self-perpetuating chain reactions. With 75% of the combined mass of potential molecular targets, protein has become a front runner in its hypothesised role it plays in oxidative stress within the cell.

Many proteins can stabilize redox metal solubilites so they become capable to redox cycle, allowing site-specific formation of very reactive products from hydrogen peroxide and superoxide (Halliwell & Gutteridge, 1999). These ROS can then mediate Fenton reactions generating protein peroxides and carbonyls along with peptide cleavage and cross-linking (Dean *et.al.*, 1993). This oxidation modification of proteins *in vivo* may affect a variety of cellular functions involving proteins, receptors, signal transduction mechanisms, transport systems and enzymes. The accumulation of oxidised proteins has been associated with many diseases such as Alzheimer's, atherosclerosis, Parkinson's, cystic fibrosis, and arthritis (Berlett & Stadtman, 1997). An important element of this hypothesis is that oxidized proteins may themselves contain reactive species which can go on further to damage more proteins and other biomolecules.

Protein hydroperoxides

Protein hydroperoxides are another product of oxidative attack on proteins, formed through a stepwise series of reactions. Oxidation of the polypeptide backbone is initiated by the subtraction of an $H^{\bullet}$ of a protein by a radical species ($R^{\bullet}$) to form a carbon-centred radical ($Pr^{\bullet}$) (*reaction vii)*. This carbon-centred radical can react with O_2

to form a protein peroxyl radical intermediate ($PrOO^{\bullet}$) *(reaction viii)*, giving rise to the protein hydroperoxides (PrOOH) *(reaction ix)*.

$$PrH + R^{\bullet} \longrightarrow Pr^{\bullet} + RH \qquad (vii)$$

$$Pr^{\bullet} + O_2 \longrightarrow PrOO^{\bullet} \qquad (viii)$$

$$PrOO^{\bullet} + e^{-} + H^{+} \longrightarrow PrOOH \qquad (ix)$$

The potential biological importance of protein hydroperoxides (PrOOHs) lies in their formation, stability and reactivity. PrOOHs have a relatively long lifetime, enabling them to diffuse a considerable distance, intracellularly and into extracellular spaces (Gebicki, 1997). This ability to transfer damage to other biomolecules has given strength to the argument that protein radicals are a relevant form of oxidative stress, and that PrOOHs themselves should be considered as ROS, capable of propagating further damage (Gebicki, 1997).

1.7.1 Protein targets of HOCl

There are many potential targets for HOCl within an amino acid, peptide or protein, including thiols, amine groups, aromatic groups and amides. The reaction of HOCl with individual amino acids has been thoroughly investigated, and the following order of reactivity with various amino acid side chains determined : Met > Cys >> cystine~His~α amino acid > Trp > Lys >> Tyr~Arg > backbone amides > Gln~Asn (Winterbourn, 1985; Pattison and Davies; 2001).

Such reaction results in side-chain modification, backbone fragmentation and cross-linking. For instance, irreversible protein crosslinks have been observed in cell membranes of red cells exposed to low doses of HOCl (Vissers *et al.*, 1998). HOCl can react with free tyrosine or tyrosine residues of proteins, resulting in the formation of 3-chlorotyrosine and 3,5-dichlorotyrosine (Domigan *et al*., 1995; Hazen *et al*., 1996; Kettle, 1996; Hazen *et al.*, 1997; Fu *et al.*, 2000).

HOCl reacts rapidly with amines present on the N-terminal of α-amino acids, peptides and proteins, as well as amine functional groups on the side chains of the amino acid residues Lys, His, and Trp to produce chloramines (Pattison & Davies, 2001). Amides react more slowly, so are relatively unimportant protein targets (Hawkins *et.al.*, 2003). However, if a large excess of HOCl is generated, reaction with backbone amides may occur. This usually results in side chain damage, protein fragmentation (Hawkins & Davies, 1998), formation of protein aggregates (Vissers & Winterbourn, 1991; Chapman, 2003) and an increased susceptibility to proteolytic degradation (Hawkins *et.al.*, 2002). HOCl can also react with the aromatic ring of Tyr, the indole ring of Trp and the guanidinium group on Arg, but these are kinetically unfavourable (Pattison & Davies, 2001).

Chloramines are generated by the reaction of HOCl with free amine group *(reaction vi).* These products can undergo secondary reactions with thiols and thiol containing proteins (Pullar *et al.*, 2000), induce radical generation that may result in protein fragmentation or lipid peroxidation (Hawkins & Davies, 1998; Hazell *et al.*, 1997) or break down to form protein carbonyls in proteins (Handelman *et al.*, 1998; Hazen *et al.*, 1998). By comparison to HOCl, chloramines have a narrower range of targets. In particular they demonstrate high reactivity with thiol groups and thioethers (R-S-R) resulting in thiol-specific protein oxidation (Peskin & Winterbourn, 2001; Grisham, 1984; Thomas, 1979).

$$RNH_2 + HOCl \longrightarrow RNHCl + H_2O \qquad (vi)$$

As chloramines are inherently unstable compounds and the products of their decomposition may be involved in toxicity to cells. Decomposition of chloramines can result in the formation of aldehydes and ketones. These are collectively termed protein carbonyls and are formed via a range of reaction mechanisms.

1.7.2 AOPPs and disease

Recently a family of oxidised protein compounds, termed “advanced oxidation protein products” (AOPPs) has emerged as a novel class of renal pathogenic mediators. AOPPs are a class of dityrosine-containing protein products formed during oxidative stress and carried mainly by albumin *in vivo* (Witko-Sarsat *et.al.*, 1996; Witko-Sarsat *et.al.*, 1998). Advanced oxidation protein products (AOPPs) were described by Witko-Sarsat et al. (1996) for the first time. They described the of presence of high levels of oxidized proteins, that they designated AOPPs, in the plasma of haemodialysed patients. Formation of AOPPs could be induced in control plasma by chlorinated oxidants such as chloramines or hypochlorous acid.

Accumulation of plasma AOPPs was first identified in patients that underwent dialysis, and was subsequently found in subjects with diabetes (Witko-Sarsat *et.al.*, 1998; Martin-Gallan *et.al.*, 2003), metabolic syndrome (Atabek *et.al.*, 2006), and non-diabetic cardiovascular disease (Witko-Sarsat *et.al.*, 1998). Recent studies have shown that chronic accumulation of plasma AOPPs significantly increases urinary protein excretion and accelerates glomerulosclerosis (scaring of the kidney tissue) (Zhou *et.al.*, 2009). An increase in AOPPs have also been shown to rapidly trigger production of intracellular superoxide by activation of NADPH oxidase leading to the up regulation of p53, Bax, caspase -3 activity and apoptosis of podcytes (Zhou *et.al.*, 2009).

HOCl-treated human serum albumin and *in vivo*-generated AOPPs can trigger oxidative bursts in neutrophils and monocytes, indicating that they can act as true inflammatory mediators (Iwao *et.al.*, 2006). More interesting is the finding that AOPPs are highly correlated to carotid intima media thickness (Drueke *et.al.*, 2002) and may be related to atherosclerotic cardiovascular events (Descamps-Latscha *et.al.*, 2005) especially with the detection of HOCl-modified proteins (including albumin) in atherosclerotic lesions (Hazell *et.al.*, 1996).

Animal studies on hypercholesterolemic rabbits have enlightened several interesting factors about AOPPs. Elevated levels of plasma AOPPs have been shown to be associated with increased atherosclerotic plaque areas (Liu *et.al.*, 2006). The mechanism by which AOPPs accelerate atherosclerosis remains to be investigated, but

it has been proposed that a possible mechanism lies with, spontaneous generation of hypercholesterolemia (Liu *et.al.*, 2006) a major risk factor for the propagation of atherosclerosis. Animal models have demonstrated that *in vivo* elevation of plasma AOPP levels resulted in excessive inflammatory response, as evidenced by increased TNF-α, enhanced macrophage invasion and SMC proliferation in the arterial wall (Liu *et.al.*, 2006). Although observational studies in animals suggest a close relationship between AOPPs and atherosclerosis, there is little evidence that AOPPs contribute to the occurrence or progression of atherosclerosis *in vivo* with relevance in humans.

1.8 Antioxidant defences

Normally, cells are able to survive in the presence of a wide range of oxidants because they possess antioxidant defences that enable them to combat the oxidant's potential to cause cellular damage and alter the redox-state of the cell. Typical mechanisms of antioxidant action include a) agents that catalytically remove ROS such as superoxide dismutase, catalase and peroxidise enzymes, b) agents that decrease ROS formation including proteins that decrease the availability of pro-oxidants such as transition metal ions, c) proteins that protect biomolecules against oxidative damage by other mechanisms and d) the physical scavenging of ROS that neutralises the radical (Halliwell & Gutteridge, 2007).

Glutathione (GSH) is the most significant low molecular weight antioxidant synthesised in cells and exists primarily in the thiol-reduced form (Meister, 1988). GSH plays a crucial role in a multitude of cellular processes such as cell differentiation, proliferation and apoptosis. Disturbances in GSH homeostasis are implicated in the progression of many human diseases including atherosclerosis (Ballatori *et al.*, 2009). The most important function of GSH appears to be as an antioxidant defence removing oxidative stress and maintaining the thiol-redox status of the cell. GSH can act as an antioxidant by directly reacting with a range of oxidants such as $OH^{\bullet}$, HOCl, $ONOO^{-}$, $RO^{\bullet}$, $NO_2^{\bullet}$ and less efficiently with $O_2^{\bullet -}$ (Halliwell & Gutteridge, 2007). GSH deficiency or a decrease in the GSH/glutathione disulfide ratio appears to result in increased

susceptibility to oxidative stress or low cellular antioxidant capacity (Ballatori *et al*., 2009).

7,8-dihydroneopterin (78NP) has been shown to exert an antioxidant effect by inhibiting oxidative damage to a variety of substrates including U937 cells (Gieseg *et al*., 2001a). The mechanism of inhibition of metal ion- and peroxyl radical-mediated LDL oxidation appear to involve scavenging of the lipid peroxyl radical, even though 78NP is a water soluble antioxidant. 78NP was further found to inhibit ROS-mediated damage to a range of other substrates and cells, including bovine serum albumin (Duggan *et al.*, 2001), erythrocytes (Gieseg *et al.*, 2001b) and the U937 monocytic cell line (Gieseg *et al.*, 2001a; Duggan *et al.*, 2002). These studies noted protection against damage that ranged from dityrosine and protein hydroperoxide formation to haemolysis, cell death and thiol oxidation.). In addition to the above substrates, 78NP has also been shown to prevent oxLDL-induced intracellular GSH loss in U937 cells by scavenging oxLDL-induced intracellular oxidants, thus maintaining the intracellular redox environment and preventing cell viability loss (Baird *et al*., 2004; Baird *et al.*, 2005)

1.9 Objectives of research

HOCl is known to oxidise the protein moiety over the lipid moiety of the LDL particle (Ermak, *et al*., 2010). This research will examine lipid and protein oxidised products following oxidation of LDL with HOCl, to access the biological importance of HOCl-oxLDL in the initiation of cell death. The research will also examine the effect of HOCl-oxLDL on cell metabolism, glutahione (GSH) levels, and cell viability. This will directly test the hypothesis that HOCl-oxLDL is toxic to cells and show whether it could be a source of intracellular stress within the plaque.

Advanced oxidation protein products (AOPPs) have been shown as a source of oxidative stress and a propogating factor in advancing kidney failure (Witko-Sarsat *et.al.*, 1998). Close correlations between pathogenesis and inflammatory conditions in uremic patients and those presenting with atherosclerosis have been observed, leading to the hypothesis that AOPPs play a causal role in the atherosclerosis. This will be

tested by creating AOPPs *in vitro* and examining the effects they exhibit on U937 cells; including measurement of cell metabolism, glutahione (GSH) levels, and cell viability.

As 78NP inhibition mechanism is thought to be through an extracellular mechanism (scavenging of oxidants released into the artery walls) it is hypothesized that 78NP will be able to inhibt the toxicity of both HOCl-oxLDL and AOPPs.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

All reagents used in this research were of analytical grade or better. All solutions were prepared using ion-exchanged ultra-filtered water, produced using a NANOpure ultrapure water system from Barnstead/Thermolyne (IA/USA).

β-mercaptoethanol	Sigma Chemical Co., Missouri, USA
1,1,3,3-Tetramethoxypropane (TMP)	Sigma Chemical Co., Missouri, USA
2-Thiobarituric acid (TBA)	Sigma-Aldrich Chemical Co., Steinheim, Germany
2,2'-azobis(amidinopropane)dihydrochloride (AAPH)	Arcos, N.J (USA)
3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT)	Sigma Chemical Co., Missouri, USA
4-Morpholine-propanesulfonic acid (MOPS)	Sigma Chemical Co., Missouri, USA
7,8-Dihydroneopterin (7,8-NP)	Schirck Laboratories, Switzerland
7-Ketocholesterol (7KC)	Sigma-Aldrich Chemical Co., Missouri, USA
Acetic acid (glacial)	Merck Ltd, Poole, England
Acetonitrile (ACN)	J.T.Baker (USA)
Acetone	Merck Ltd, Poole, England
Ammonium ferrous sulfate	Hopkin and Williams Ltd, Essex
Argon gas	BOC Gases, Auckland, New Zealand
Bicinchoninic acid (BCA) protein determination kit	Pierce, Illinois, USA
Bovine serum albumin (BSA) (fraction V- fatty acid free)	Gibco Invitrogen Corporation, Auckland, New Zealand
Bromophenol blue	Sigma Chemical Co., Missouri, USA
Butylated hydroxytoluene (BHT)	Sigma Chemical Co., Missouri, USA
Chelex 100 resin	Bio-Rad Laboratories, California, USA
Cholesterol reagent	Roche Diagnostics, USA
Chloroform	Merck and Univar

Copper chloride ($CuCl_2$)	Sigma Chemical Co., Missouri, USA
Diethyl ether	Merck, Darmstadt, Germany
Dihydroethidium (DHE)	Invitrogen,, Oregon, USA
Dimethyl sulphoxide (DMSO)	AnalaR, Poole, England
Ethanol	Merck, Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma Chemical Co., Missouri, USA
Glutathione (reduced form)	Sigma Chemical Co., Missouri, USA
Glycerol	Sigma Chemical Co., Missouri, USA
Hexane	Mallinckrodt Chemicals, New Jersey, USA
Hydrochloric acid, fuming 37 % (HCl)	Merck, Darmstadt, Germany
Isopropanol	Mallinckrodt Chemicals, New Jersey, USA
Lipoprotein Electrophoresis Kit	Beckman Coulter, USA
Methanol	Merck, Darmstadt, Germany
Molecular Weight Marker	Fermentas International Inc, Ontario, Canada
Monobromobimane (MBB)	Fluka Analytical, Switzerland
Nitrogen gas	BOC Gases, Auckland, New Zealand
NuPAGE 4-12% Bis-Tris Gel, 1.0 mm x 10 well	Invitrogen, California, USA
Phenol	Sigma Chemical Co, Missouri, USA
Phosphoric acid (H_3PO_4)	Merck, Darmstadt, Germany
Potassium bromide (KBr)	Merck, Darmstadt, Germany
Potassium hydroxide (KOH)	Merck, Darmstadt, Germany
Sodium acetate ($C_2H_3NaO_2$)	Merck, Darmstadt, Germany
Sodium chloride (NaCl)	Merck, Darmstadt, Germany
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_20$)	Scharlau Chemie, Italy
Sodium dodecyl sulphate (SDS)	Sigma Chemical Co., Missouri, USA
Sodium hydrogen carbonate ($NaHCO_3$)	Merck, Darmstadt, Germany
Sodium hydroxide (NaOH)	Scharlau Chemie, Italy
Sodium hypochlorite (NaOCl)	Jasol Dynawhite, Christchurch, New Zealand
Sulfuric acid (H_2SO_4)	BDH lab supplies Ltd, Poole, England
Trichloroacetic acid (TCA)	Merck, Darmstadt, Germany
Trypan blue solution (0.4%)	Sigma Chemical Co., Missouri, USA
Xylenol orange	Sigma Chemical Co, Missouri, USA

2.1.2 Media

Earle's balanced salt solution (EBSS)	Invitrogen, California, USA
Foetal bovine serum (FBS)	Invitrogen, California, USA
Penicillin/Streptomycin (10000 units/ml penicillin G and 10000 µg/ml streptomycin)	Gibco Invitrogen, Auckland, New Zealand
Roswell Park Memorial Institute (RPMI) -1640 media, with phenol red	Sigma-Aldrich Chemical Co., Missouri, USA
Roswell Park Memorial Institute (RPMI) -1640 media, without phenol red	Sigma-Aldrich Chemical Co., Missouri, USA

2.1.3 General solutions, media and buffers

2.1.3.1 Phosphate buffered saline (PBS*)*

Phosphate buffered saline (PBS), containing 150 mM sodium chloride and 10 mM sodium dihydrogen orthophosphate (pH 7.4) was vacuum filtered through a 0.45 µm Phenex filter membrane (Phenomenex). If required for cell culture, the PBS solution was sterilized by autoclaving (15 minutes, 121ºC and 15 psi) and stored at 4ºC. PBS was warmed in a water bath to 37ºC prior to use with cells.

2.1.3.2 Roswell Park Memorial Institute (RPMI)-1640 media (with or without phenol red)

The media was prepared as per manufacturer's instructions. Powdered RPMI-1640 (with or without phenol red) was dissolved in nanopure water, followed by addition of sodium bicarbonate and pH adjustment to 7.4 with 1 M NaOH. The media was filter-sterilised using a peristaltic pump (CP-600, Life Technologies) and a 0.20 µm Sartolab®-P20 filter (Sartorius AG, Goettingen, Germany) into sterile bottles. Media was stored at 4ºC and warmed to 37ºC in a water-bath before use.

2.1.3.3 Bovine serum albumin (BSA) solutions

Bovine serum albumin – fraction V, fatty acid free (BSA) stock solution (7.5 mg/ml) was prepared fresh, and used within one week, by dissolving BSA in filter sterilized PBS. Solution was stored under argon at 4 ºC and warmed to 37 ºC in water-bath before use.

2.1.3.4 7,8-Dihydroneopterin (78NP) solution

A 2 mM stock of 78NP (MW= 255.2 g/mol) was prepared fresh, immediately before each experiment. 78NP was dissolved in degassed ice cold RPMI-1640 medium during a 5 -10 minute sonication. 78NP solution was subsequently filter-sterilised using a 0.22 µm MS® PES syringe filter (Membrane Solutions, USA) and diluted to working concentrations in warm RPMI-1640 media.

2.2 Methods

2.2.1 Cell culture

All cell experiments were carried out under aseptic conditions in a Class II biological safety cabinet (Clyde-Apex BH 200). Sterile plastic wares were supplied by Falcon, Terumo, Unomedical and Greiner Bio-one. Media and solutions were sterilised either by autoclaving or by filtration through a sterile 0.22 µm membrane filter. All equipment and tissue culture items were sprayed thoroughly with 70% ($^{v}/_{v}$) ethanol before being transferred to the class II biological safety cabinet. Cells were maintained at 37°C in a humidified incubator, with an atmosphere calibrated to 5% carbon dioxide: 95% air (Sanyo CO_2 Incubator).Viable cells were counted using a haemocytometer and a light microscope after staining with trypan blue solution at a ratio of 1:1.

2.2.1.1 Cell culture media

RPMI-1640 medium (with phenol red) containing 100 units/ml penicillin G and 100 µg/ml streptomycin was supplemented with 5% ($^{v}/_{v}$) foetal bovine serum for culturing U937 cells. This growth media is referred to in the thesis as U937 culture medium.

2.2.1.2 Preparation of U937 cell line

The U937 cell line was originally developed from the pleural fluid of a 37-year old man with generalised histiocytic lymphoma (Sundstrom & Nilsson, 1976). Our U937 cells were a gift from the Haematology Research Laboratory at the Christchurch School of Medicine, University of Otago. A 1 ml vial containing 20 x 10^6 cells/ml was removed from liquid nitrogen storage and defrosted in a 37ºC water-bath until almost completely

thawed. The concentrated cell suspension was poured into 30 ml of RPMI-1640 medium at 37°C in a 50 ml centrifuge tube and centrifuged at 500 g for 5 minutes to separate the DMSO freezing medium and cells. The resulting cell pellet was re-suspended in 10 ml of RPMI-1640 medium in a 25 cm^2 tissue culture flask. Cell density was maintained at 0.4-1.5 x 10^6 cells/ml by passaging in Cellstar® 75 cm^2 tissue culture flasks (Greiner Bio-one) every 2-3 days.

2.2.1.3 Cell experiment procedures

All cell experiments were performed using Cellstar® 12-well suspension culture plates (Greiner Bio-one), which were coated with 8 µl of 5% bovine serum albumin (BSA) per well, (exception being experiments with treatments involving BSA, oxidised or not), to prevent cells adhering to the plastic in the absence of FBS. A 5% BSA solution was made up in RPMI-1640 (without phenol red) immediately before each experiment. Viable cells were counted using a haemocytometer under a light microscope after staining with trypan blue at a ratio of 1:1. The required quantity of cells was pelleted by centrifugation at 500 g for 5 minutes at room temperature and re-suspended in RPMI-1640 (with or without phenol red) at 37 ºC, to a concentration of 1 x 10^6 cells/ml. The cell suspension was then aliquoted into wells containing RPMI-1640 (with or without phenol red) and any reagents specific to the experiment, to give a final concentration of 0.5 x 10^6 cells/ml.

2.2.2 Blood collection and plasma preparation

With ethics approval from Upper South A Ethics Committee, CTY/01/04/036, human blood was drawn from healthy volunteers following an overnight fast. Blood was drawn by venipuncture using a 21G x $^3/_4$ inch or 19 G needle attached to a 30 ml syringe (Terumo, USA), subsequently it was transferred into 50 ml polypropylene conical tubes containing 0.5 ml of 100 mg/ml EDTA (pH 7.4)

Whole blood was centrifuged at 4,100 g for 20 minutes at 4ºC with the brake off to separate red blood cells and plasma. The resulting top yellow plasma was subsequently transferred to SS34 rotor centrifuge tubes and centrifuged at 11,000 g for 30 minutes at 4ºC, with slow acceleration/deceleration, to remove remaining cellular debris in the

plasma. Plasma from all donors was then pooled together into a single measuring cylinder, to minimise inter-individual variation. Plasma was stored at -80ºC in 32 ml aliquots for up to 6 months until required.

2.2.3 LDL preparation

2.2.3.1 Extraction of LDL from plasma

LDL isolation utilised a Beckman Near Vertical Rotor and employed the method of Chung *et al.* (1980) with modifications described by Gieseg and Esterbauer (1994), which was directly adapted from Dr. Wendy Jessup (Heart Research Institute Ltd, Sydney) for LDL preparation in a vertical rotor. The protocol requires a single gradient that redistributes during ultracentrifugation to form a density gradient, which separates lipoproteins.

A 32 ml tube of frozen human plasma was defrosted under cold running water and centrifuged at 4700 rpm for 10 minutes at 4ºC to pellet precipitated fibrinogen. The supernatant was decanted into a beaker and placed immediately on ice. Potassium bromide (KBr) was gradually dissolved in plasma, to a final concentration of 382 mg KBr/ml plasma, altering the plasma density to 1.24 g/ml. The solution was stirred gently to prevent foam formation, which is an indication of LDL denaturation. Plasma was maintained on ice and under argon gas until ultracentrifugation.

Eight millilitres of 1 mg/ml EDTA (pH 7.4) was added to each of 8 OptiSeal™ polyallomer centrifuge tubes (Beckman Coulter, USA) before under-layering with 4 ml of KBr-plasma, using a needle attached to a 5 ml syringe. Ultracentrifuge tubes were transferred to the NVTi-65 rotor and centrifuged at 60,000 rpm for 2 hours at 10ºC with slow acceleration/deceleration using an Optima™ L-90K Preparative Ultracentrifuge (Beckman Coulter, Inc., Fullerton, California). Following centrifugation, a yellow/orange coloured band of LDL in the density range of 1.019 – 1.063 g/ml was collected using a 20 ml syringe attached to a 90º-bend needle

2.2.3.2 Determination of cholesterol content of LDL

The cholesterol content of LDL was determined by enzymatic cholesterol assay using a CHOL kit (Roche Diagnostic). One millilitre of cholesterol reagent was incubated with 10 µl of LDL at room temperature for 10 minutes. The absorbance was read at 500 nm against a blank containing only cholesterol reagent.

LDL concentration was then calculated from the absorbance, based on the estimate that cholesterol accounts for 31.69% of the entire LDL particle, by weight, and that LDL has a molecular weight of 2.5 kDa (Gieseg and Esterbauer, 1994).

Calculation: absorbance x 14.9 = [cholesterol] (nM)

[cholesterol] (M) x 386.64 g/mol = [cholesterol] (g/l)
[cholesterol] (M) x 100/31.69 = g/1 LDL or [LDL] (mg/ml)

2.2.4 LDL washing and dilution

2.2.4.1 Dialysis tubing treatment

Dry dialysis tubing (Medicell International, UK) with 25 mm flat width and molecular weight cut off (14,000 Daltons), was cut into 22 cm lengths, and boiled for 20 minutes on a heating block in a glass beaker containing a solution of 5% $^{w}/_{v}$ $NaHCO_3$ and 1mM EDTA. Following washing with distilled water, the tubes were boiled again in a glass beaker containing distilled water. After 20 minutes of boiling, the tubes were washed thoroughly with distilled water and stored in 50% ethanol at 4ºC.

2.2.4.2 LDL washing

Ten millilitres of LDL was transferred to a piece of dialysis tubing secured at one end by a double knot, and closed at the top with a single knot. An Eppendorf tube was clipped to the top of the dialysis tubing to make it buoyant. LDL was dialysed against three 1 litre changes of sterilized PBS (section 2.1.3.1) over a 24 hour period at room temperature. LDL was diluted to a final concentration of 2.5 mg/ml (total mass) before any subsequent manipulations or use in experiments, by the addition of sterile PBS

based on the concentration calculated after dialysis (section 2.2.3.2). If no further manipulations were to be made to the LDL, it was filter-sterilised using a 0.22 µm syringe filter and stored at 4ºC in the dark, under argon gas.

2.2.5 Measurement of hypochlorite (HOCl) concentration

The concentration of sodium hypochlorite (NaOCl), stored at 4°C, was measured using the method described by van den Berg and Winterbourn (1994). A pH 12 buffer containing 25 ml of 0.2 M KCl and 6 ml of 0.2 M NaOH diluted to 100 ml with nanopure water was prepared. The buffer was then used to dilute the stock NaOCl 500-fold, and the absorbance at 292 nm measured on a Shimadzu UV-1601PC UV-visible spectrophotometer (Shimadzu Corporation, Japan). The concentration was determined using the extinction coefficient (350 $M^{-1}cm^{-1}$) of HOCl at pH 12 (Gazda and Margerum, 1994).

2.2.6 LDL oxidation

2.2.6.1 HOCl oxidation of LDL

Oxidation was induced by incubating LDL (2.5mg/ml) in the presence of 42 mM HOCl at the following molar oxidant/protein ratios; 250/1, 500/1, 1000/1, 1500/1 and 2000/1. Solutions were warmed to 37 ºC before mixing, and then placed for 30 minutes in a shaking incubator (Bioline, Edwards Instrument Company, Australia) in the dark. Temperature was maintained at 37 ºC and had an orbital motion at a speed of 118 rpm in which the constant motion ensured oxygenation of the solutions. After oxidation the hypochlorite-oxidised LDL (referred to herein as HOCl-oxLDL) was filter-sterilised using a 0.22 µm syringe filter and stored at 4ºC in the dark, under argon gas.

2.2.6.2 Copper oxidation of LDL

LDL at 10 mg/ml (total mass) was transferred to a section of dialysis tubing in the same manner as LDL washing (section 2.2.4.2). Fifty millimolar copper chloride ($CuCl_2$) solution was combined with the LDL inside the dialysis tubing to give a final concentration of 0.5 mM $CuCl_2$. The LDL-containing dialysis tubing was placed in a

bottle containing 1 L PBS/50 mg LDL, plus $CuCl_2$ at a final concentration of 0.5 mM $CuCl_2$. LDL was dialysed against 0.5 mM $CuCl_2$ in PBS overnight at 37ºC in a heated orbital shaker.

The dialysis tubing containing copper-oxidised LDL (referred to herein as Cu-oxLDL) was then transferred to a fresh bottle containing 1 L of PBS and stirred with 1 g of washed Chelex-100 at 4ºC for two hours. This was repeated twice, with the final incubation taking place overnight. Cu-OxLDL was filter-sterilised using a 0.22 µm syringe filter and stored at 4ºC.

2.2.7 OxALB oxidation

Oxidised BSA was prepared from a stock solution of 7.5mg/ml BSA (see section 2.1.3.3) with the addition of sterile PBS (see section 2.1.3.1) and 42 µM HOCl at the following molar oxidant/protein ratios; 250/1, 500/1, 1000/1, and 2000/1, to give a final concentration of 5 mg/ml BSA. Solutions were warmed to 37ºC before mixing, and then placed for 30 minutes in an orbital incubator (Bioline, Edwards Instrument Company, Australia) in the dark. Temperature was maintained at 37ºC and had an orbital motion at a speed of 118 rpm in which the constant motion ensured oxygenation of the solutions. After oxidation the hypochlorite-oxidised BSA (referred to herein as oxALB) was filter-sterilised using a 0.22 µm syringe filter and stored at 4ºC in the dark, under argon gas and used within 2 weeks.

Finalised albumin oxidation method

Following observations made on initial experiments, the oxidation of BSA was altered slightly, to remove chloramines. OxALB was prepared as mentioned above, but underwent dialysis treatment before filter sterilization.

After oxidation in the orbital incubator oxALB solutions were dialysed (*section 2.2.4.2*), against PBS, at room temperature for 4 hours, to remove any excess HOCl. The dialysis tubing was subsequently transferred to a fresh 1 L bottle of fresh PBS containing 2-fold excess of methionine and dialysed overnight. Following the overnight dialysis, oxALB was further dialysed against a fresh 1 L bottle of PBS for 4 hours,

before it was filter-sterilised using a 0.22 µm syringe filter and stored at 4ºC in the dark, under argon gas and used within 2 weeks. All experiments using this finalised method of oxidation have been noted in the legends of the figures as met-oxALB.

2.2.8 Cell viability assays

2.2.8.1 Trypan Blue Exclusion Staining

Trypan blue exclusion staining measures cell viability by analysing cell membrane integrity (Moldeus *et al.*, 1978). Viable cells have intact membranes that are impermeable to the dye and, as a consequence, appear opaque when viewed under a microscope. Dead cells have compromised membrane integrity and stain a deep blue colour due to trypan blue uptake.

U937 cells were mixed with the stain at 1:1 ratio, and 20 µl added to a haemocytometer (Marienfeld, Germany). Subsequently, a light microscope was used to count the number of opaque (alive) cells in defined regions of a haemocytometer. The proportion of viable cells in that well was calculated by dividing the number of viable cells by the total number of cells (both alive and dead).

2.2.8.2 The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay

A) MTT assay solutions

Five mg/ml of 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) was prepared in RPMI 1640 without phenol red. This solution was filter-sterilised using a 0.22 µm MS® PES syringe and stored at -20 ºC in dark until use.

10% ($^{w}/_{v}$) sodium dodecyl sulphate (SDS) in 0.01 M hydrochloric acid (HCl) was stored at room temperature.

B) MTT assay

The MTT reduction assay measures cell viability by quantifying metabolically active cells through their ability to reduce the MTT reagent. This laboratory has previously

shown that results produced by this method agree with results obtained by the trypan blue exclusion assay when used on U937 and THP-1 cells (Baird *et.al.*, 2004). Metabolically active cells can endocytose and metabolise the yellow MTT compound, via the action of cellular NADPH dehydrogenases, into a insoluble purple MTT-formazan product that can be solubilized and quantified spectrophotometrically (Mosmann, 1983). The colour intensity provides an indication of the concentration of cells and their metabolic activity.

Following experimental treatment, U937 cells from each well were washed with warm PBS and incubated in 1ml of RPMI-1640 medium (without phenol red) containing 0.5 mg/ml MTT for 2 hour. The insoluble purple crystals were then dissolved by mixing with 1 ml of 10% ($^{w}/_{v}$) SDS. The absorbance was read at 570 nm, against a blank lacking cells but containing all other reagents.

2.2.9 PrOOH determination by acetic acid-FOX assay

2.2.9.1 Solutions for FOX assay

A) Xylenol orange solution

5mM xylenol orange was prepared by dissolving xylenol orange (MW 760.6g/mol) in 25mM sulfuric acid for the acetic acid-FOX assay. The solution was to be used within one month.

B) Ammonium ferrous sulphate solution

5mM ammonium ferrous sulfate was made by dissolving ammonium ferrous sulphate (MW 392.16 g/mol) in 25mM sulfuric acid for the acetic acid-FOX assay. The solution was to be used within one week.

C) 2,2'-azobis(amidinopropane)dihydrochloride (AAPH) solutions

A stock solution of 250mM was prepared the day of experiment by dissolving AAPH (MW 271.19 g/mol) in chilled PBS and stored on ice until required.

2.2.9.2 PrOOH formation in BSA and LDL

PrOOHs were measured directly after HOCl insult on LDL and BSA described above (section 2.2.6.1 and 2.2.7 respectively) using the ferric-xylenol orange (FOX) assay described (section 2.2.9.3).

PrOOHs were also measured after oxidation of BSA with 10 mM 2,2'-azobis(amidinopropane)dihydrochloride (AAPH). A 5 mg/ml BSA solution was incubated with or without AAPH in an incubator (Bioline, Edwards Instrument Company, Australia) with a temperature and the orbital motion set to 37 ºC and 118 rpm respectively. At set time points 1 ml samples were removed and PrOOH formation was measured using the FOX assay described below (section 2.2.9.3).

2.2.9.3 Acetic acid-FOX assay

The acetic acid-FOX assay is a modified version of the sulphuric acid-FOX assay, as developed by Joseph Pearson (2002). This assay was used to detect PrOOHs produced in oxidised (lipo)proteins.

One millilitre samples (1 mg/ml) were extracted in triplicate, into 1.7 ml microtubes where 140 µl of 72% TCA was added and then samples were chilled on ice in the dark for 5 minutes. This was followed by centrifugation at 10000 rpm at 4ºC for 5 minutes. Supernatent was discarded and pellets washed in 1 ml of 1:1 methanol/chloroform (or 5% TCA for oxALB samples) prior to centrifugation at 10000 rpm at 4ºC for 5 minutes. Supernatent was again discarded and resulting protein pellets were dried by inverting microtubes for 5 minutes. The pellets were then dissolved in 900 µl of 50% acetic acid, if needed pellets were also broken up using a flame-sealed Pasteur pipettes and vortexed.

Once completely dissolved, 50 µl of 5 mM xylenol orange and 50 µl of 5 mM ammonium ferrous sulphate were added and samples incubated in the dark, on ice, for 30 minutes. Following incubation the samples were centrifuged at 10,000 rpm at 4ºC for 5 minutes. Afterwards the samples were transferred to couvettes and their absorbance read against a water blank at 560 nm. Concentration of PrOOHs in samples were quantified as a rise in PrOOHs formation compared to controls, consisting of 900

µl of 50% acetic acid, 50 µl of xylenol orange and 50 µl of ammonium ferrous sulphate, using the extinction co-efficient for calculating PrOOH concentrations in LDL and BSA is 35,500 $M^{-1}cm^{-1}$.

2.2.10 HPLC analyses

High Performance Liquid Chromatography (HPLC) is a widely used system for separating, identifying and quantifying compounds. The HPLC system (Shimadzu Corporation, Japan) was used for a number of analyses and comprised of a controller (LC-20AD), a fluorescence detector (RF-10AXL), a UV-Vis detector (SPD-M20A), an auto sampler (SIL-20AC HT), a column oven (CTO-20A), inline vacuum degasser (DGU-20A5), and a communication bus module (CBM-20A). Peak areas were quantified using the LCSolution software package (version 1.22 SP1, 2002-2006) .

All the mobile phases used were sonicated (Alphatech Systems Ltd & Co., Auckland) for 15 minutes prior to pumping through the HPLC system. Analytes in samples were standardised against pure standards with known concentrations to determine their concentrations.

2.2.10.1 Protein tyrosine residue oxidation

A) Acid hydrolysis

Tyrosine residue oxidation serves as an indicator of protein damage by free radical exposure (Gieseg *et al.*, 1993; Gieseg *et al.*, 2003). In this research, tyrosine residue loss upon HOCl reaction with to LDL and BSA, was examined by measuring tyrosine residue levels after acid hydrolysis of proteins and HPLC analysis.

For the acid hydrolysis of proteins, 200 µl of sample, HOCl-oxLDL or oxALB (diluted to 1 mg/ml in nanopure H_2O), was placed in triplicate, in glass Durham tubes (7.5 mm x 50 mm Biolab), and dried down under vacuum for approximately 2 hours. The Durham tubes were then placed into a Pico-Tag vial (Millipore, USA) containing 1 ml of 6M HCl with 1% (w/v) phenol and 50 µl of β-mercaptoethanol. Argon gas was flushed through the Pico-Tag vial for 5 minutes to remove oxygen. The vial was then evacuated by connecting to a high vacuum line for 2 seconds (the Vac. gauge pressure was

maintained below 200 mm Torr). Subsequently, the vial was incubated in a 110ºC oven for 16 hours, and allowed to cool to room temperature before opening. The tubes were removed from the vials and dried by centrifugation under vacuum in a Speed Vac for 2 hours before re-solubilising the pellet with 200 µl of 0.1% trifluoroacetic acid (TFA) (pH 2.5). The sample was then centrifuged at 20,800 g for 5 minutes at 4ºC before transferring 100 µl of the resulting supernatant to an auto sampling vial.

Ten µl was injected onto a reverse phase Aqua C18, 250 x 4.6 mm, 5µm column (Phenomenex). The column was developed with mobile phase A (0.1% (v/v) TFA at pH 2.5) and mobile phase B (100% acetonitrile), pumped at a flow rate of 1 ml/minute. TFA with a concentration of 0.1% (v/v) at pH 2.5 was prepared by adding 1 ml of TFA to 800 ml of nanopure water, adjusting the pH to 2.5 with 10 M NaOH, and making up to a final volume of 1 L. Tyrosine and DOPA, were detected by a fluorescence detector using excitation and emission wavelengths set at 280 nm and 320 nm, respectively. Dityrosine was measured by changing the emission wave length to 410 nm after the elution of tyrosine. A comparison of the peaks and elution time produced by a tyrosine (MW 181.2) standard of 1 µM concentration, DOPA (MW 197.19) and dityrosine (MW 360.361) standards of 5 µM concentration, made up in 0.1% (v/v) TFA (pH 2.5), were used to quantify the elution time and concentration of tyrosine, DOPA and dityrosine within the samples.

2.2.10.2 Intracellular GSH analysis

Monobromobimane (MBB) is a cell-permeable fluorescent dye that binds thiol groups, that can be used for the detection of GSH. MBB alkylates thiol groups to form GSH-MBB adducts which can then be detected by the HPLC system after protein precipitation (Cotgreave and Moldeus, 1986). All procedures involving MBB were performed under minimum exposure of light since MBB is light sensitive.

A 40 mM stock of MBB was prepared by dissolving MBB (MW 271.1 g/mol) in acetonitrile, which was stored at 4ºC in the dark for up to 2 weeks. Reduced GSH (MW 307.3 g/mol) was dissolved in cold PBS, to 5 and 10 µM concentrations, immediately prior to HPLC analysis for use as standards. Mobile phase A was 0.25% acetic acid and mobile phase B was 100% acetonitrile.

After removal of the incubation medium by centrifugation, cells were washed twice in warm sterile PBS and resuspended in 1.5 ml Eppendorf tubes in, 400 µl of PBS, 9 µl of 0.1 M NaOH (to increase the pH to 8), and 10 µl of 40 mM MBB, in this order. After 20 minutes of incubation in the dark at room temperature, 20 µl of 100% (w/v) trichloroacetic acid (TCA) was added to lyse the cells and precipitate cellular proteins. The cell lysate was collected and centrifuged at 10000 rpm for 5 minutes at 4ºC to pellet cellular proteins. One hundred microlitres of the resulting supernatant was transferred to auto sampling vial inserts and 5 µl injected onto the Phenosphere reverse phase C-18, 150×4.6 mm, 5 µm column (Phenomenex, Auckland, NZ), heated to 35°C.

GSH-MBB adducts were detected by the fluorescence detector with excitation and emission wavelengths set at 394 nm and 480 nm, respectively. Mobile phase A (consisting of 0.25% acetic acid in nanopure water) and mobile phase B (consisting of 100% acetonitrile) were pumped through the column at a flow rate of 1.5 ml/minute with the following gradient program:

Time (minutes)	Mobile phase A	Mobile phase B
0	90%	10%
10	90%	10%
11	0%	100%
15	95%	5%
16	95%	5%
20	95%	5%

2.2.10.3 Simultaneous detection of 7-Ketocholesterol (7KC) and α-Tocopherol

Detection of free 7KC was performed using a modification of the method described by Kritharides *et al*., (1993). Mobile phase was ACN/isopropanol/H2O in a ratio of 44:54:2. Lipid extraction of the samples was carried out by the addition of 10 µl of 20 mg/ml BHT, 20 µl of 100 mg/ml EDTA, 500 µl ethanol and 2 ml of hexane, before vortexing for 60 seconds. Samples were then centrifuged at 200 g for 10 minutes at 4ºC to obtain complete phase separation. Fourteen hundred microlitres of the top hexane

layer was removed, transferred to a tapered glass test tube and dried completely under nitrogen gas.

Esterified 7KC was quantified by measuring both free and total 7KC in a sample and calculating the difference. Detection of total 7KC was performed using a lipid extraction (as above), followed by alkaline hydrolysis that was used to release esterified 7KC before detection.

After evaporation with nitrogen gas, the remaining residue was resolubilised in 2 ml of 20% ($^{w}/_{v}$) KOH in methanol and 2.5 ml of diethyl ether. The sample was then flushed with argon gas and vortexed thoroughly. Samples were incubated on ice for 3 hours, vortexing every 30 minutes before the reaction was stopped by the addition of 2 ml of 20% acetic acid. Two and a half millilitres of hexane was added and samples were vortexed for 60 seconds before transferring 4 ml of the upper layer into a tapered glass test tube and evaporating completely under nitrogen gas.

The residues, free and total, were resolubilised in 100 µl of resolubilising phase (ACN/isopropanol in a ratio of 4:5). One hundred microlitres of the resulting supernatant was transferred to a glass auto sampling vial insert and 20 µl was injected onto the Phenosphere reverse phase C-18, 250 x 4.6 mm, 5 µm column (Phenomenex, Auckland, NZ) which was heated to 35 ºC. Analysis of 7KC was performed by detecting 234 nm absorbance. Mobile phase was ACN/isopropanol/H_2O in a ratio of 44:54:2.

Low molecular weight molecule, α-tocopherol, scavengers oxidants such HOCl, and can be detected simultaneously in the samples prepared by the 7KC method described above. Analysis of α-tocopherol was performed by detection of at an excitation wavelength of 295 nm and emission wavelength of 325 nm.

2.2.10.4 Thiobarbituric acid reactive species (TBARS) analysis

The TBARS assay provides a means of quantifying general lipid peroxidation. The method used is an adaptation of that described by Draper *et al.*, (1993). When combined, 2-thiobarbituric acid (TBA) and the maldondialdehyde (MDA) lipid

hydroperoxide break-down product readily react, forming the pink coloured TBA-MDA adduct which is fluorometrically detected by HPLC.

The MDA standard was prepared on the day of analysis by the dilution of 6.07 M 1,1,3,3-tatramethoxypropane (TMP) in ethanol/water (2:3), with subsequent dilution in water to the desired concentration. The mobile phase was 50 mM sodium dihydrogen phosphate, with the pH adjusted to 6.8 using NaOH, and combined with methanol in a ratio of 65:35. A 42 mM 2-thiobarbituric acid (TBA) solution was made fresh on the day of analysis by dissolving in nanopure water on a hot plate, ensuring that the temperature did not exceed 55ºC.

Samples were diluted with PBS to 1 mg/ml, before 200 µl of this solution was transferred to a 1.7 ml microtube. To this, 100 µl of 50 mM phosphoric acid and 20 µl of 20 mg/ml BHT in methanol, were added and vortexed to mix. Then 100 µl of the above TBA reagent was added before incubating on a shaking heater block (Eppendorf Thermomixer 5436) at 95ºC for 30 minutes. Samples were then placed directly onto ice and allowed to cool before centrifugation at 10,000 rpm for 10 minutes at 4ºC.

One hundred microlitres of the remaining supernatant was transferred to an autosampling vial insert and 20 µl was injected onto the Phenosphere reverse phase C-18, 50 x 4.6 mm, 5 µm column (Phenomenex, Auckland, NZ) which was heated to 30ºC. TBARS were detected using excitation and emission wavelengths of 525 nm and 550 nm, respectively. The mobile phase was methanol and 50 mM sodium dihydrogen phosphate (pH 6.8) in a ratio of 45:55. The concentration of TBARS was quantified by comparison with the peak areas of 0 and 1 µM MDA standards.

2.2.11 Flow cytometry

Flow cytometry is a powerful technique that simultaneously measures and analyses multiple characteristics of single cells, such as cell size, granularity and relative fluorescence intensity, as they flow in a fluid stream through a beam of light. It gives high-throughput (for a large number of cells) automated quantification of fluorescence instead of producing fluorescent images.

Cells were probed with DHE or annexinV/PI for intracellular superoxide or apoptosis detection as (see section 2.2.11.1 and 2.2.11.2). Cell samples were then re-suspended in 500 µl of PBS in Eppendorf tubes, and analyzed using the Accuri$^{@}$ C6 flow cytometer (BD Biosciences, USA). DHE-probed and annexinV/PI probed cells were detected using the 585/40 band-pass (BP) filter in FL-2 and 530/30 BP filter in FL-1, respectively. Ten thousand cells were analyzed in a sample gated to remove subcellular debris. All data were collected and analyzed using the cFlow Plus software.

2.2.11.1 Dihydroethidium (DHE)

Dihydroethidium (DHE) has been widely used for detecting intracellular superoxide anions. This cell permeable dye reacts with superoxide resulting in the formation of a two-electron oxidised product, ethidium (E^{+}), which binds to DNA and leads to enhancement of red fluorescence (λex/λem = 510/605 nm) (Budd *et al.*, 1997; Zhao *et al*., 2003). DHE (MW 315.4) stock was prepared in DMSO and stored at -20ºC.

Following experimental treatment and subsequent removal of incubation medium, cells were washed in warm PBS and resuspended in, with 150 µl of 10 µM DHE, dissolved in sterile PBS, for 20 minutes. Cells were then again washed in PBS twice to remove excess DHE. Cell pellets were resuspended in 500 µl PBS and examined by flow cytometry (see section 2.2.11).

2.2.11.2 Apoptosis analysis by Annexin V/PI

Apoptosis is a distinct event that triggers characteristic morphological and biological changes in the cellular life cycle. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. AnnexinV labelled with a fluorescent tag, is a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS. Since AnnexinV staining precedes the loss of membrane integrity which accompanies the later stages of cell death resulting from either apoptotic or necrotic processes, staining with AnnexinV was used in conjunction with a live/dead dye propidium iodide (PI) to allow identification of early apoptotic cells (PI negative, AnnexinV positive) from dead cells (PI positive, AnnexinV positive).

Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI.

Following experimental treatment and subsequent removal of incubation medium, cells were washed in binding buffer and resuspended in 100 µl of binding buffer containing 2 µl annexinV, 5 µl PI and incubated in the dark for 15 minutes. After incubation samples were topped up to 500 µl with binding buffer and examined by flow cytometry (see section 2.2.11).

2.2.12 SDS-PAGE analysis

2.2.12.1 Solutions for SDS-PAGE analysis

Cracker buffer was prepared by dissolving SDS (MW = 288.38 g/mol), glycerol (MW = 92.10 g/mol), bromophenol blue (MW = 670.02 g/mol) in 0.5 M Tris-HCl in nanopure water (pH 6.8) and made up to a final volume of 50 ml. Prior to use, 1 ml of the above solution was mixed with 20 µl of β-mercaptoethanol and 2 ul of 100 mg/ml ethylenediaminetetraacetic acid (EDTA). The final cracker buffer hence consisted of 0.125 M Tris-HCl (pH 6.8), 1% ($^{w}/_{v}$) SDS, 20% ($^{w}/_{v}$) glycerol, 0.1% ($^{w}/_{v}$) bromophenol blue, 2% ($^{v}/_{v}$) β-mercaptoethanol, and 0.5 mM EDTA.

Lysis buffer consisted of 40 mM of HEPES (MW = 238.31 g/mol), 50 mM of NaCl (MW = 58.44 g/mol), 1 mM EDTA (MW = 372.24 g/mol), and 1 mM EGTA (MW = 380.4 g/mol) in nanopure water, with pH adjusted to 7.4 using 10 M NaOH and stored at 4°C. Prior to use, Complete, Mini protease inhibitor stock (7×) was added to lysis buffer. The 7× stock protease inhibitor solution was prepared as per manufacturer's instructions. One Complete, Mini protease inhibitor cocktail tablet (Roche, Germany) was dissolved in 1.5 ml of nanopure water to give a 7× stock solution. The completed lysis buffer was stored on ice until use.

MOPS (4-morpholine-propanesulfonic acid) buffer consisted of 50 mM MOPS, 50 mM Tris base, 0.1% ($^{w}/_{v}$) SDS, and 1 mM EDTA in nanopure water, with the pH adjusted to 7.7 by adding concentrated HCl.

2.2.12.2 Cell processing of U937 cells for SDS-PAGE analysis

After experimental treatment, cell samples were collected and washed twice with PBS by centrifugation at 500 g for 5 minutes at room temperature. One hundred and fifty µl of ice cold complete lysis buffer was used to lyse U937 cell pellet. The samples were then vortexed and placed on ice to ensure thorough lysing of the cells for at least 15 minutes.

After protein analysis, the volume of cell lysate containing 75 µg of protein was transferred to a new 1.7ml Eppendorf tube. Ice cold acetone was then added to precipitate the proteins (ratio of cell lysate:acetone 1:10). After incubation on ice for 5 minutes, the sample was centrifuged at 20,800 g for 5 minutes at 4°C. The resulting protein pellet was dissolved in "cracker" buffer for SDS-PAGE analysis (see section 2.2.12.1).

2.2.12.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Cracker buffer (30 µl) was used to dissolve the acetone-precipitated protein pellet described above to give a final protein concentration of 2.5 µg/µl. The sample was then heated to 95 ºC in a heating block for 3 minutes and centrifuged for 5 minutes at 20,800 g at room temperature to remove any cell debris.

A gradient polyacrylamide gel, 4-12% (Bis-Tris Gel, Invitrogen, Carslband, CA, USA), was placed in the XCell SureLock™ Mini-Cell system (Invitrogen, US) and the MOPS running buffer added to the buffer reservoir. Five microliters of Fermentas pre-stained molecular weight marker mix (Fermentas International Inc, Canada) and 20 µL of samples (containing 50 µg proteins) were loaded into the wells of the gel. The gel was run at 100V for 15mins to stack the gel before electrophoresis at 200 V for approximately 1 hour until the loading buffer dye reached the bottom of the gel.

Statistical analysis

Data were graphed and analysed statistically using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, California, USA). Significance was confirmed via a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Significant levels are indicated in the following manner: (*) $p \leq 0.05$, (**) $p \leq 0.01$ and (***) $p \leq 0.001$.

Results displayed in this thesis are taken from a representative of three separate experiments, unless otherwise stated in the legend. The mean and standard error of the mean (SEM) shown within each experiment were calculated from triplicate samples in every case. Some results, flow cytometry, are the combined result of several separate experiments, with the mean and SEM shown and calculated across those experiments. This will be stated in the legend.

3. RESULTS

3.1 Characterisation of the modification of low-density lipoprotein (LDL) by hypochlorous acid (HOCl)

The majority of previous studies exploring cellular effects of oxLDL have been carried out using human oxLDL oxidised *in vitro* in the presence of metal ions such as Cu^{2+}. Previous studies in our laboratory have shown that in U937 cells, copper oxidised LDL (Cu-oxLDL) kills cells through excessive oxidant production resulting in the loss of intracellular anti-oxidant, inactivation of key metabolic enzymes and necrosis. Recently, interest has arisen in other forms of oxLDL, with potential relevance to atherosclerosis.

Only in the past ten years has research begun into the mechansism of HOCl induced oxidation of LDL (HOCl-oxLDL), and its toxicity to cells. Ermak *et.al,* 2010 and Vicca *et.al,* 2003 have shown certain apoptotic pathways are activated in response to HOCl-oxLDL.

Characterisation of the biochemical properties of HOCl-oxLDL was the first step taken in understanding how HOCl oxidises the LDL molecule and how it may play a role in the toxicity of cells within an atherosclerotic inflamation site.

3.1.1 Tyrosine loss on oxidised LDL

It has been observed that the majority of the oxidant, HOCl, is consumed by reaction with proteins, with lipid peroxidation being a secondary reaction (Hawkins *et.al.*, 2002). Kinetic evidence shows that the reaction occurs in amino acid side-chains with the order of reactivity being Met > Cys >> Cystine ~ His ~ α amino > Trp > Lys >> Tyr ~ Arg > Gln ~ Asn (Malle *et al.*, 2006). To investigate whether the protein component of the LDL molecule was oxidised, determination of the quantity of protein-bound tyrosine remaining on LDL after oxidation was assessed. Tyrosine was chosen as a representative amino acid because it is low on the reactivity series. Thus one could assume that if tyrosine had become oxidised, then the majority of other amino acids

were also being oxidised. After oxidation of the LDL molecule with HOCl at varying oxidant/protein molar ratios, the amount of protein-bound tyrosine remaining was quantified though acid hydrolysis and subsequent analysis with HPLC (figure 3.1). Significant reductions in the levels of tyrosine were observed with increasing molar excesses; at the lowest molar ratio (500/1) 64.4 % of native levels were lost, reaching a maximum loss of 89.98% induced by the highest molar ratio of 2000/1.

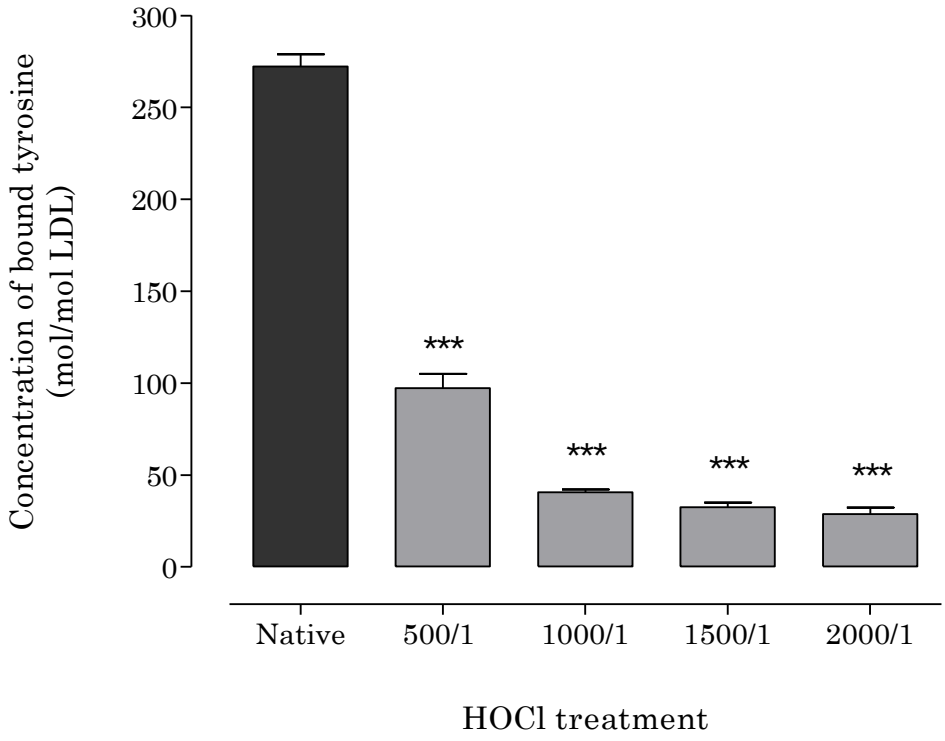


Figure 3.1 Concentration of bound tyrosine to LDL protein. Native LDL (2.5 mg/ml) was oxidised following incubation with HOCl at 37℃ for 30 minutes at concentrations corresponding to oxidant/protein molar ratios of 500/1, 1000/1, 1500/1 and 2000/1. The concentration of bound tyrosine was determined by HPLC analysis. Significance is indicated from the respective native LDL control.

3.1.2 α-Tocopherol loses its protective ability in excess HOCl

α-Tocopherol is a well characterised antioxidant within LDL with high affinity for HOCl. It readily reacts with HOCl preventing it from doing so with other molecules. However in excess- HOCl all the α-tocopherol become oxidised and therefore are unable to prevent oxidation of the LDL molecule. Of interest was the relationship between the loss of tyrosine residues and α-tocopherol levels, which mimicked a near identical rate of loss with increasing molar ratios of HOCl. Significant reductions in the levels of α-tocopherol were observed with increasing molar excesses. The anti-oxidant lost its protective ability as it became oxidised. Even at the lowest molar ratio (500/1), 69.5 % of native levels were lost, reaching a maximum loss of 92.7% induced by the highest molar ratio of 2000/1. These results were graphed together with the tyrosine oxidation data (figure 3.1) to show the comparative rate of loss between protein-bound tyrosine and α-tocopherol (figure 3.2).

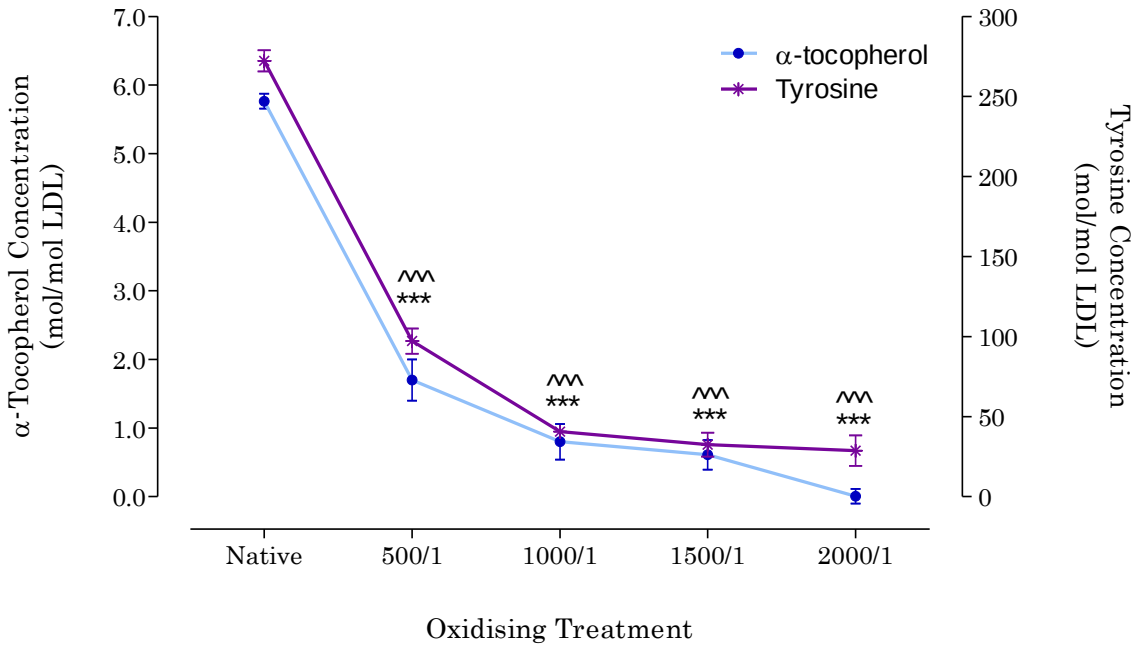


Figure 3.2 Concentration of tyrosine and α-Tocopherol after LDL oxidation. Native LDL (2.5 mg/ml) was oxidised following incubation with HOCl at 37℃ for 30 minutes at concentrations corresponding to oxidant/protein molar ratios of 500/1, 1000/1, 1500/1 and 2000/1. The concentration of tyrosine and with α-tocopherol was determined through HPLC analysis of 200 µl samples of native and oxidised LDL. Significance is indicated from the respective native LDL control (^^^ for tyrosine, *** for α-tocopherol).

3.1.3 Protein oxidation of LDL

Protein hydroperoxides are another product of oxidative attack on proteins, which have the ability to transfer oxidative damage to other biomolecules (Gebicki *et.al.*,1997). The kinetics of protein hydroperoxide (PrOOH) formation on LDL were investigated by incubating 1 µM LDL with 10 mM AAPH at 37ºC for 4 hours (figure 3.3) using the acetic-acid FOX assay. After an initial lag phase of 2 hours PrOOHs increased rapidly at an approximate rate of 15 moles PrOOH/moles LDL per hour, before tapering off at 40 moles PrOOH/moles LDL. Aside from confirming previous findings regarding the kinetics of PrOOH formation, the following experiment aimed to establish the relative levels of PrOOH formed when using a radical generator such as AAPH, in order to compare to a parallel experiment using HOCl.

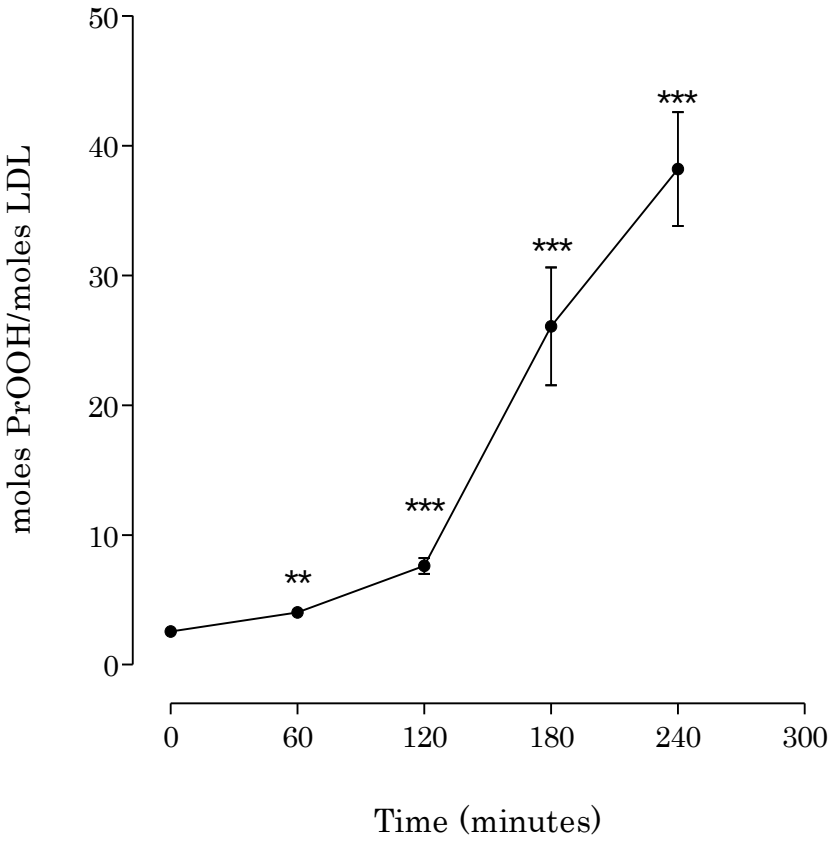


Figure 3.3 PrOOH formation on apoB when LDL was oxidised by 10 mM AAPH. LDL was incubated with 10 mM AAPH at 37ºC on an orbital shaker. Samples were removed at set time points and analysed for protein hydroperoxides using the acetic acid-FOX assay. PrOOHs were adjusted against a blank control containing FOX reagents and 50 % acetic acid. Significance is indicated from the 0 hour time point control.

HOCl is highly reactive (within 30 - 40 minutes); therefore measurement of PrOOH kinetics is very difficult. Instead, PrOOH formation on LDL with HOCl was investigated by measuring final concentrations of PrOOH immediately after oxidation, or on two week old oxidation solutions that had been stored under argon at 4 ºC.

PrOOH formation increased on samples analysed immediately after oxidation (figure 3.4a), in an almost linear manner with increasing oxidant/protein molar ratios. There was a significant ($P < 0.05$) 7-fold increase from native levels at the 500/1treatment, with the biggest increase of 2 moles/moles LDL at 2000/1 treatment. Over time in an oxygen-deprived environment PrOOH formation was still occurring at a very slow rate (figure 3.4b). PrOOHs increased by approximately 1 mole/mol LDL compared to their respective measurements two weeks prior, except for the 500/1 treatment which received a 2.2 mol/mol LDL increase.

However, even with the increase in PrOOHs after two weeks, the rise from native levels was insignificant when compared to the amount formed using the radical generator AAPH (figure 3.3).

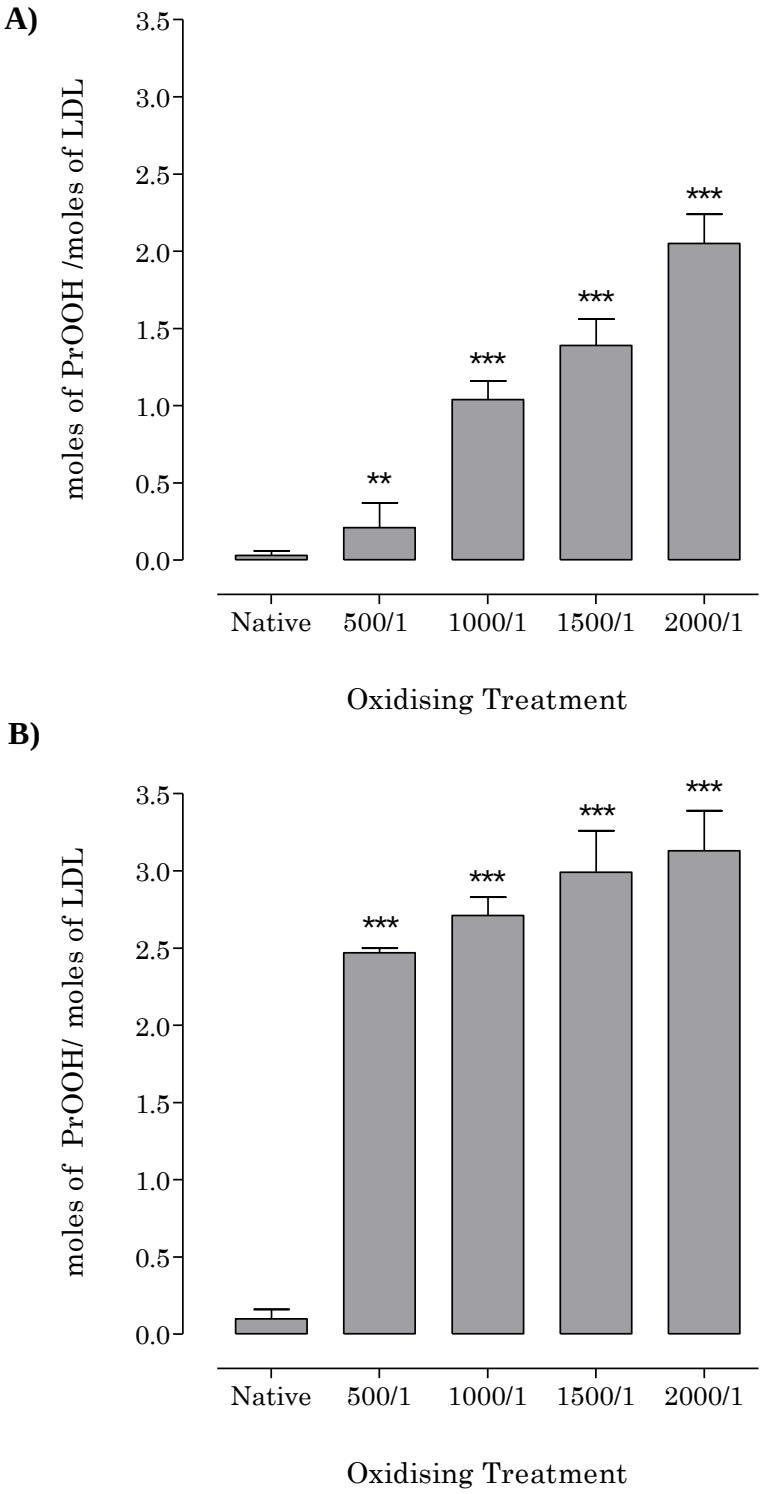


Figure 3.4 PrOOH formation on apoB when LDL was oxidised by varying HOCl treatments. LDL was incubated at 37℃ with HOCl for 30 minutes. Samples were removed immediately after oxidation **(A)**, or removed after storage under argon for two weeks **(B)**; and analysed for protein hydroperoxides using the acetic acid-FOX assay. PrOOHs were adjusted against blank controls containing FOX reagents and 50 % acetic acid. Significance is indicated from the respective native LDL control.

3.1.4 Thiobarbaturic Acid Reactive Species (TBARS) produced during the oxidation of LDL

LDL contains 22% protein (apoB-100), 22% phospholipids, 8% cholesterol, 42% cholesteryl esters, and 6% triglycerides (wt/wt) (Orlova *et.al.*, 1999). Since it was determined that HOCl interacted with the protein component of LDL through the oxidation of amino acids and the formation of PrOOHs, the next obvious question was; is HOCl affecting not only the protein moiety but the lipid moiety as well? Lipid peroxidation is studied extensively in relation to not only disease, but also modulation of antioxidants and other contexts. A large number of by-products are formed during this process which can be measured by various assays. The most common method used is the estimation of aldehydic products by their ability to react with thiobarbaturic acid (TBA) to form a pink coloured TBA-MDA adduct which is fluorometrically detected by HPLC.

Across the various HOCl-oxLDL treatments, there was no significant increase in TBARS compared to native levels in unmodified LDL. However there was a significant increase in levels found in native LDL 0.15 ± 0.004 compared to Cu-oxLDL with to 9.35 ± 0.100 mol of TBARS/mol LDL (figure 3.5).

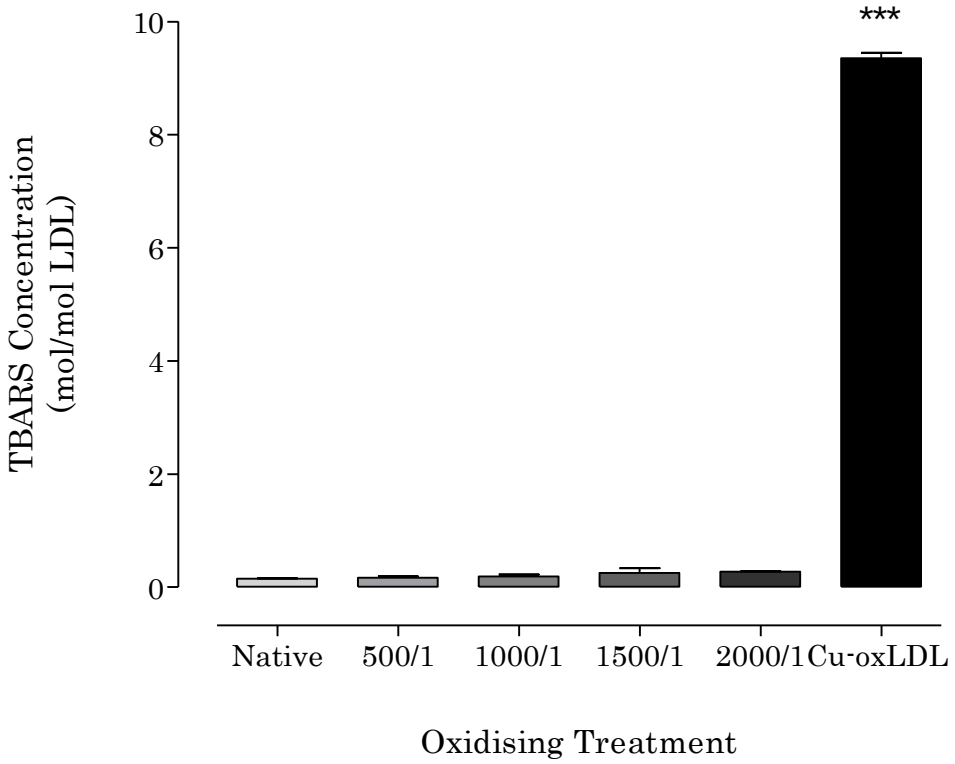


Figure 3.5 Concentration of Thiobarbaturic Acid Reactive Species (TBARS) produced by the oxidation of LDL with HOCl. Native LDL was oxidised following incubation at 37℃ with either; HOCl for 30 minutes at concentrations corresponding to oxidant/protein molar ratios of 500/1, 1000/1, 1500/1 and 2000/1 or for 24 hours with 0.5 mM $CuCl_2$. TBARS analysis was carried out on samples taken from the oxidised LDL solutions (2.5 mg/ml) and diluted 1:5 in nanopure water. Significance is indicated from the native LDL control.

To investigate whether LDL would oxidise further in the presence of contaminating free metal ions in RPMI medium and absence of α-tocopherol (see figure 3.2), HOCl-oxLDL was incubated in 12 well culture plates. Two hundred microlitres of HOCl-oxLDL was incubated with 800 µl of RPMI (no phenol red), at 37℃ in a CO_2 incubator (5.0%). TBARS analysis was carried out on samples taken before and after incubation for 24 hours (figure 3.6). There was surprisingly significant reductions ($P < 0.001$) in TBARS in samples analysed after the 24 hour incubation period across at all treatments, this suggests that the initial trace amounts of TBARS found after oxidation were being lost or degraded.

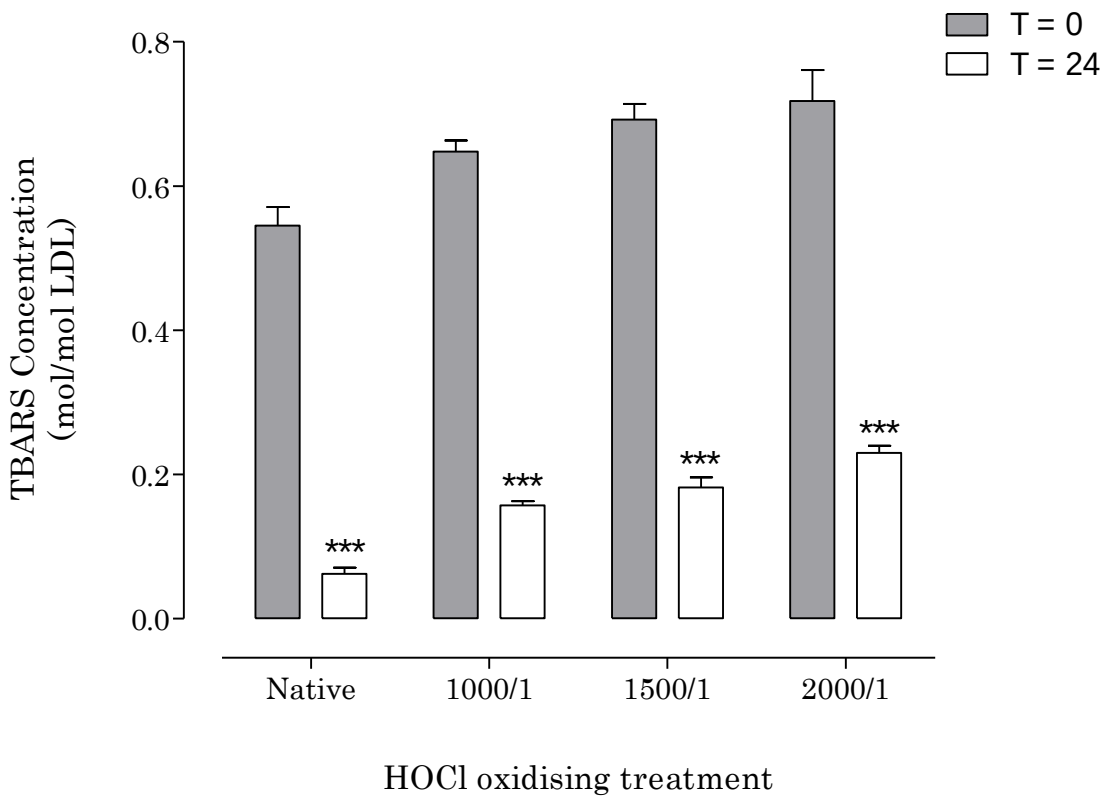


Figure **3.6 Concentration of Thiobarbaturic Acid Reactive Species (TBARS) in oxLDL before and after incubation with RPMI-1640.** Native LDL (2.5 mg/ml) was oxidised following incubation at 37℃ with HOCl for 30 minutes at concentrations corresponding to oxidant/protein molar ratios of 500/1, 1000/1, 1500/1 and 2000/1. Using 12 well culture plates, 200 µl of oxLDL was incubated in 800 µl RPMI-1640, at 37℃ in a CO_2 incubator (5.0%) for 24 hours. TBARS analysis was carried out on 200 µl samples taken from wells before and after 24hr incubation. Significance is indicated between the respective before and after samples at each treatment.

It has been shown that HOCl has the ability to break down malondialdehyde (MDA) (Winterbourn *et.al.*, 1993). This may explain why the TBARS were degraded after prolonged exposure, and even as to why only trace amounts were detected in the initial experiment (see figure 3.3). If this is the case then this does not suggest that lipid peroxidation is not occurring, simply that the validity of the assay in these circumstances should be questioned, and another means of lipid peroxidation measured.

3.1.5 7-Ketocholesterol levels in oxLDL compared to Cu-oxLDL

7-ketocholesterol (7KC) is the major product of cholesterol oxidation and used as a biomarker for lipid peroxidation and cytotoxicity. In accordance with previous work in this lab, these results (figure 3.7) demonstrate that up to 88% of the total intracellular 7KC detected was esterified. Note that total 7KC represents combined esterified and non-esterified 7KC. These levels significantly increased with higher HOCl/LDL molar ratios. This effect was most pronounced at the 2000/1 treatment, where there was an 84% increase in total 7KC relative to the respective native control.

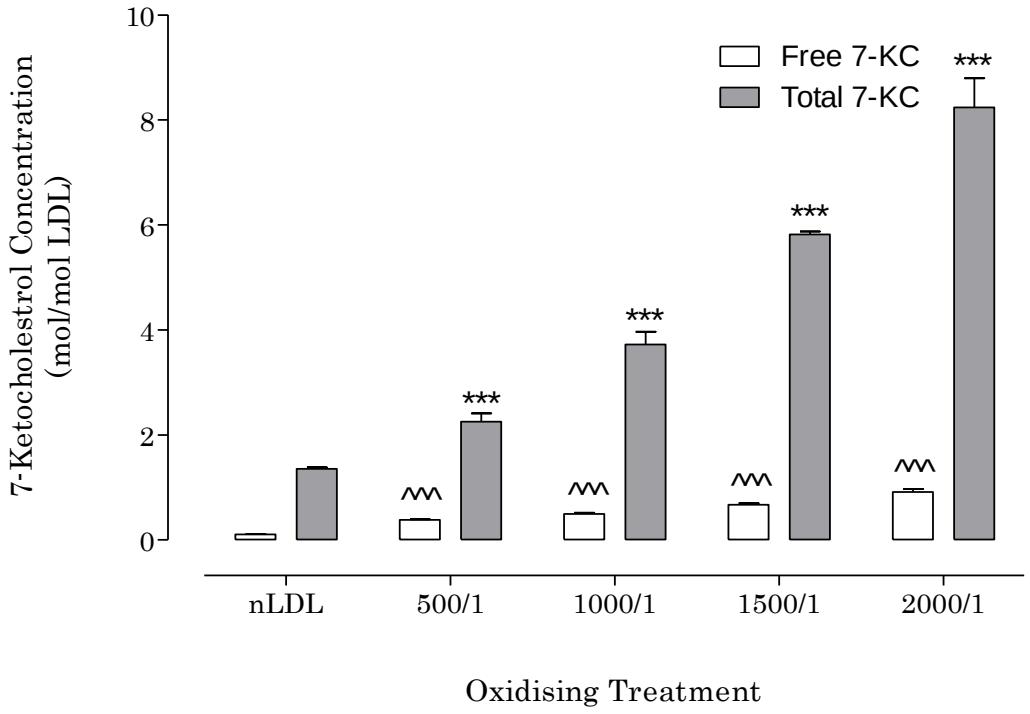


Figure 3.7 Concentration of free and total 7-Ketochoelsterol (mol/mol LDL) after LDL oxidation. Native LDL (2.5 mg/ml) was oxidised following incubation with HOCl at 37℃ for 30 minutes at concentrations corresponding to oxidant/protein molar ratios of 500/1, 1000/1, 1500/1 and 2000/1. The concentration of 7KC was determined via HPLC analysis. Significance is indicated from the respective native LDL control (^^^ for free 7KC, *** for total 7KC)

A parallel experiment was conducted to compare 7KC formation in HOCl-oxLDL and Cu-oxLDL (figure 3.8). Unlike the preceding experiment there was no significant increase in 7KC levels as HOCl/LDL molar ratios were increased. However, there was a 180-fold increase from native levels to those produced with Cu-oxLDL. Overall levels of 7KC were minute in the HOCl-oxLDL treatments compared to Cu-oxLDL, with a 29 fold increase from the highest amount produced in the 2000/1 HOCl-oxLDL treatment.

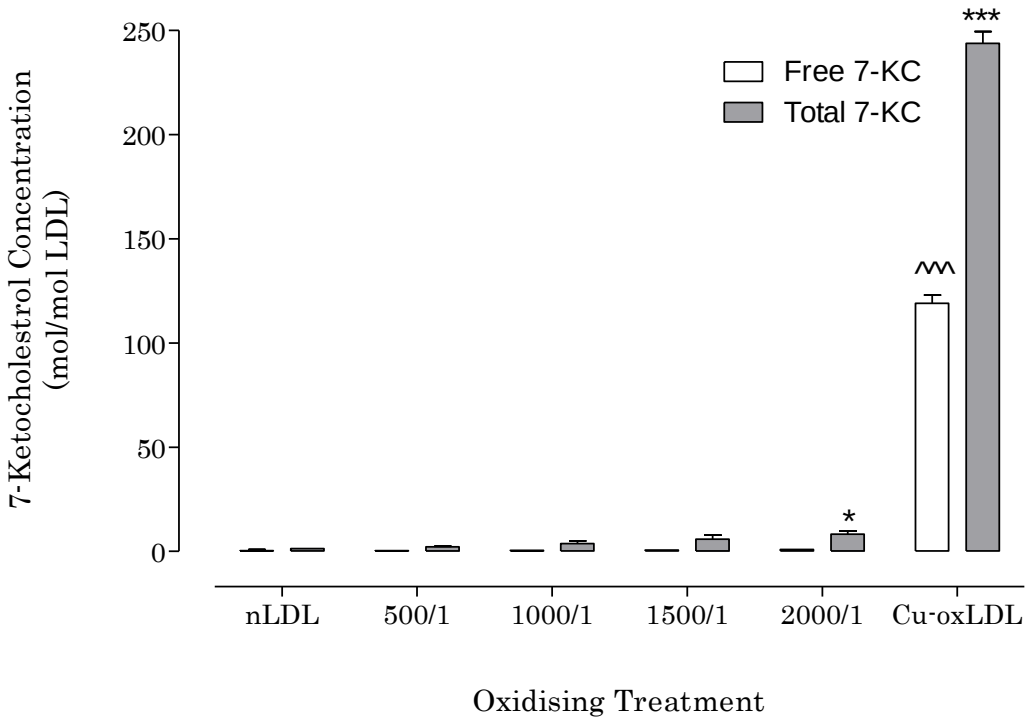


Figure 3.8 Concentration of free and total 7-Ketochoelsterol after LDL oxidation. Native LDL was oxidised following incubation at 37℃ with either; HOCl for 30 minutes at concentrations corresponding to oxidant/protein molar ratios of 500/1, 1000/1, 1500/1 and 2000/1 or for 24 hours with 0.5 mM $CuCl_2$. The concentration of 7KC was determined with HPLC analysis. Significance is indicated from the respective native LDL control (^^^ for free 7KC, *** for total 7KC).

3.1.6 Simultaneous detection of 7-Ketocholesterol and α-Tocopherol in oxLDL

Both 7KC and α-Tocopherol were measured simultaneously following oxidation of LDL with HOCl at varying oxidant/protein molar ratios. 7KC increased in an approximately linear manner with a rise in 7KC detected as HOCl concentration increased. α-Tocopherol levels were lost almost immediately with a significant ten-fold reduction at 500/1 treatment compared to control, with none detected at any of the higher protein/oxidant molar ratios. This pattern was reflected in both the free (figure 3.9a) and total (figure3.9b) samples.

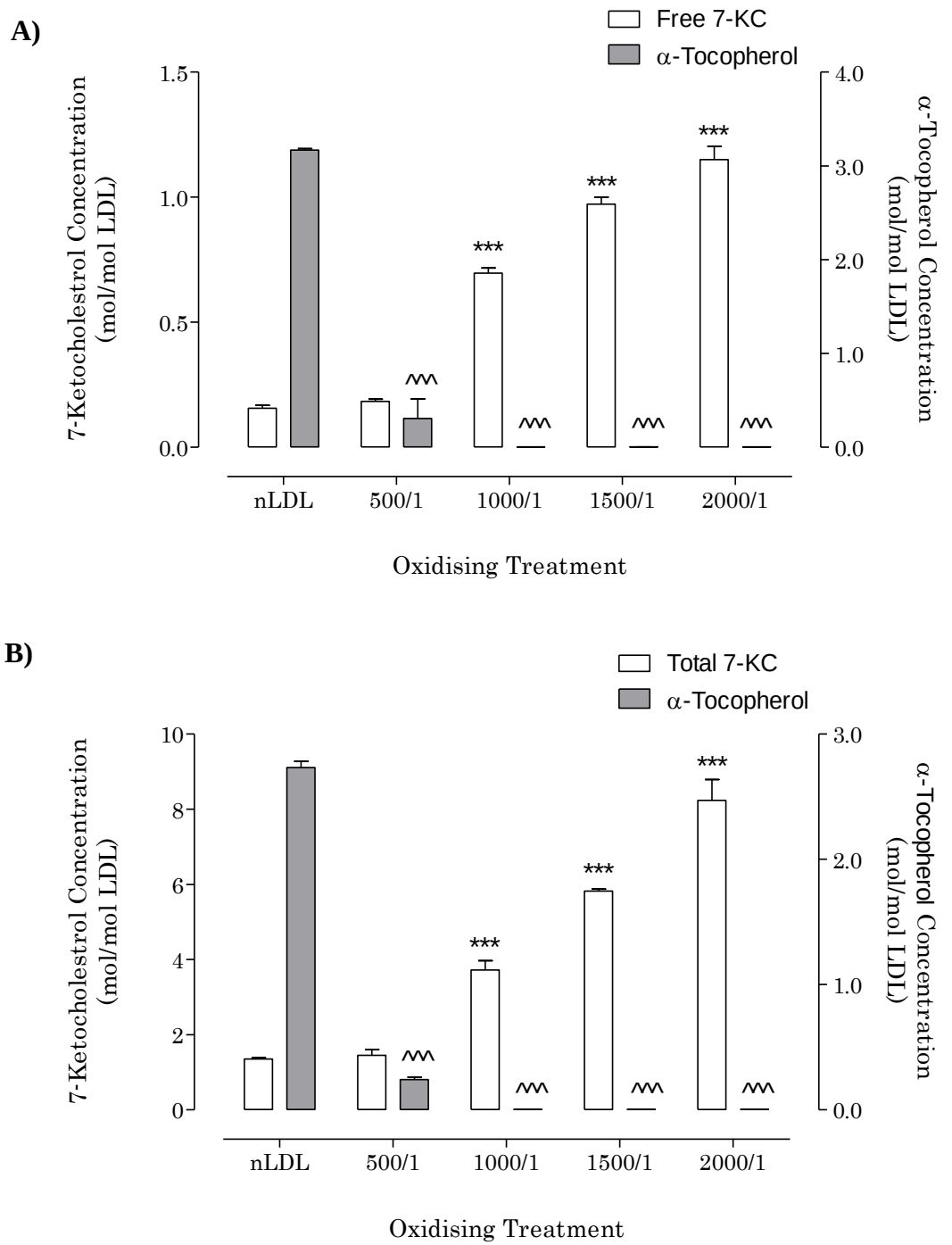


Figure 3.9 Concentration of 7-Ketochoelsterol (7KC) and α-Tocopherol on LDL molecule upon oxidation with HOCl. LDL was oxidised following incubation with HOCl at 37℃ for 30 minutes at varying oxidant/protein molar ratios. The concentration of free 7KC and α-tocopherol **(A)** and total 7KC with α-tocopherol **(B)** was determined with HPLC analysis. Significance is indicated from the respective native LDL control on both graphs (^^^ for free 7KC, *** for α-tocopherol).

3.1.7 Measurement of 7-ketocholesterol before and after incubation in RPMI

To further assess whether HOCl-oxLDL would oxidise in the presence of contaminating free metal ions in the RPMI medium and after the loss of α-tocopherol, HOCl-oxLDL was incubated with RPMI (no phenol red) for 24 hours at 37℃ (figure 3.10). Subsequently 7KC was measured, and found that there were significant increases, across all treatments, between samples taken before and after incubation. The most significant increase (1.5 fold) occurred at the 500/1 treatment.

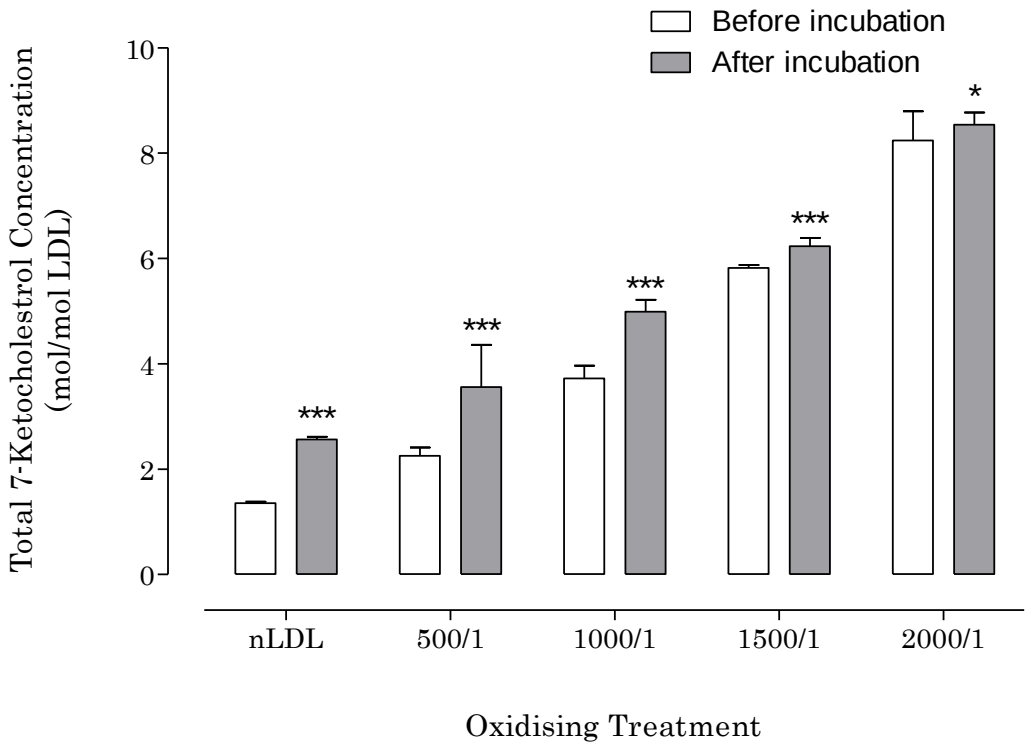


Figure 3.10 Total 7KC before and after incubation in RPMI medium (no serum) for 24 hours. Native LDL was oxidised following incubation at 37℃ with HOCl for 30 minutes at concentrations corresponding to oxidant/protein molar ratios of 500/1, 1000/1, 1500/1 and 2000/1. Using 12 well culture plates, 200 µl of the samples were incubated in 800 µl RPMI-1640 at 37℃ in a CO_2 incubator (5.0%). Total 7KC analysis was carried out on 200 µl samples taken from wells before and after the 24 hour incubation. Significance is indicated between the respective before and after samples of each treatment.

7-ketocholesterol correlates strongly with toxicity. Elevated levels of 7KC are thought to contribute to the increasing toxicity of oxidised LDL. With such low levels found in HOCl-oxLDL compared to Cu-oxLDL, the question arises; is HOCl-oxLDL even toxic to cells like its metal ion oxidised counterpart?

3.2 Toxicity of HOCl-oxLDL

To investigate the potential role of HOCl-oxLDL-induced cytotoxicity, the following set of experiments began with examination of the effects of HOCl-oxLDL on the U937 human monocyte-like cell line before characterising the nature of the cytotoxic mechanism triggered by the HOCl-oxLDL. Based on previous studies we expected this to involve the generation of oxidative stress leading to cell death. This should be clearly demonstrated by the loss of glutathione and the inhibition of cell death by the antioxidant 7,8-dihydroneopterin. Aside from confirming previous findings regarding its toxicity, these experiments aimed to establish appropriate concentrations of HOCl-oxLDL at the best oxidant/protein molar ratio for use in later experiments.

3.2.1 Concentration dependant toxicity with HOCl-oxLDL on U937 cells

The toxicity of HOCl-oxLDL to U937 cells was examined by exposing cells to increasing concentrations of HOCl-oxLDL in RPMI-1640 cell medium for 24 hours. Cell viability was subsequently measured using the MTT reduction assay (figure 3.11). U937 cells displayed a concentration-dependent decrease in cell viability following incubation with HOCl-oxLDL. There was no significant difference in cell viability between increasing oxidant/protein ratios, rather there was a concentration dependent loss of viability with increasing levels of HOCl-oxLDL given to cells. Final HOCl-oxLDL concentrations of 0.25, 0.5, and 1.0 mg/ml, at any given HOCl/LDL ratio, reduced cell viability by approximately 29, 39, and 52% of the control, respectively.

The precise toxicity of HOCl-oxLDL to U937 cells was found to vary slightly between different batches prepared, with the median lethal dose (LD_{50}) between 0.75 -1.0 mg/ml HOCl-oxLDL. The LD_{50} of Cu-oxLDL is known to vary from one batch to another although the exact cause has not been identified (Gieseg *et al*., 2009). The least variation occurred at 1000/1 molar ratio, and so it was decided that subsequent experiments would be conducted at 1.0 mg/ml with the 1000/1 treatment, unless otherwise stated.

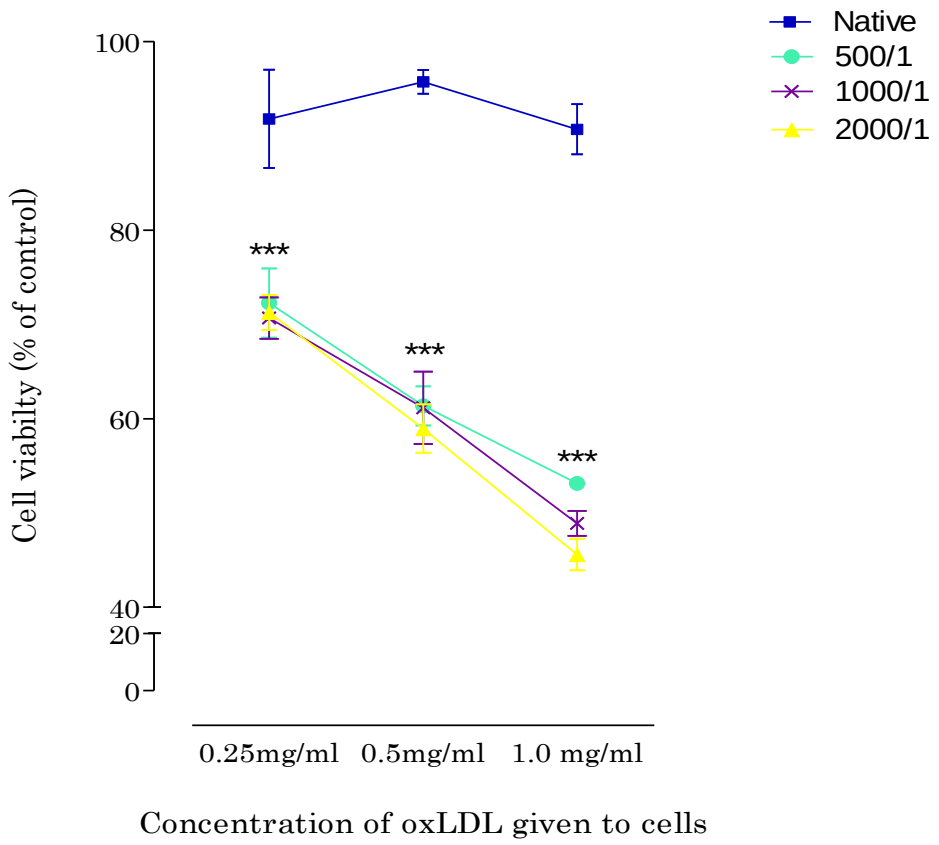


Figure 3.11 Viability loss of U937 cells following exposure to different concentrations of HOCl-oxLDL with varying HOCl treatments for 24 hours. LDL (2.5 mg/ml) was incubated at 37℃ with HOCl for 30 minutes at concentrations corresponding to oxidant/protein molar ratios of 250/1, 500/1, 1000/1, and 2000/1. U937 cells (0.5 x 10^6 cells/ml) in RPMI-1640 were incubated with varying concentrations of HOCl-oxLDL for 24 hours, and the results analysed by MTT assay. Viability was measured as a percentage of a control containing the same cell treatment but with PBS in place of native or oxLDL. Significance is indicated between the respective concentrations of oxLDL given to cells (*** depicts for all treaments 500/1, 1000/1, 2000/1).

The following experiment (figure 3.12) further illustrates that there is no effect of increasing protein/oxidant molar ratios on the toxicity of HOCl-oxLDL. LDL was oxidised with increasing concentrations of HOCl to give the following oxidant/protein ratios 250/1, 500/1, 1000/1 and 2000/1. HOCl-oxLDL (1.0 mg/ml) was incubated with U937 cells in RPMI for 24 hours at 37℃ and subsequently measured for viability using the MTT reduction assay. Cell viability decreased by approximately 55% compared with the cell only control (containing 0.5 x 10^6 cells/ml, PBS and RPMI) at all oxidising treatments.

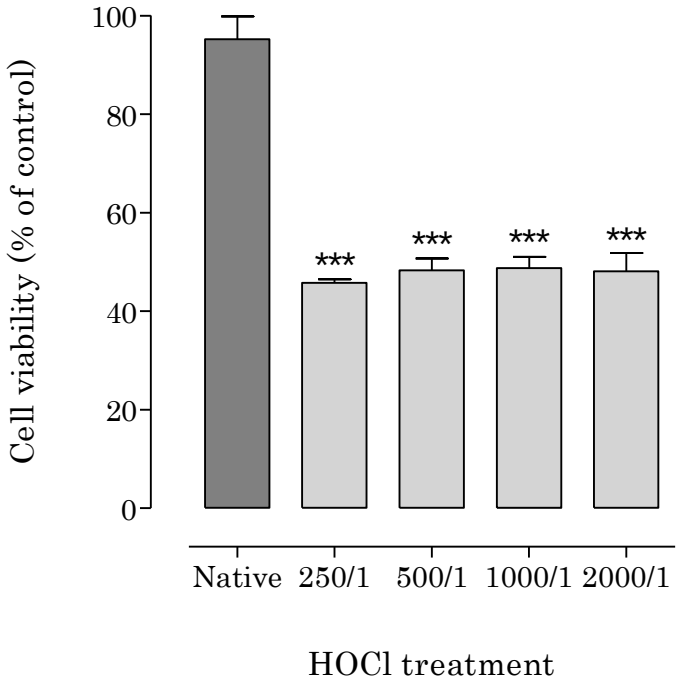


Figure 3.12 Effect of HOCl-oxLDL with varying HOCl treatments on U937s after 24 hours. LDL (2.5 mg/ml) was incubated at 37℃ with HOCl for 30 minutes at concentrations corresponding to oxidant/protein molar ratios of 250/1, 500/1, 1000/1, and 2000/1. U937 cells (0.5 x 10^6 cells/ml) in RPMI-1640 were incubated with 1.0 mg/ml HOCl-oxLDL for 24 hours, and the results analysed by MTT assay. Viability was measured as a percentage of a control containing the same cell treatment but with PBS in place of native or oxLDL. Significance is indicated from the native LDL control.

3.2.2 Effect of chloramines on the toxicity of oxLDL

HOCl reacts readily with amines to form chloramines which are known to be toxic to cells (Thomas *et.al.*, 1985). To investigate whether chloramines were responsible for the toxicity of HOCl-oxLDL they were removed during the dialysis step using excess methionine (figure 3.13). Cells were incubated with or without HOCl-oxLDL (0.5 and 1.0 mg/ml) that had either been treated or not treated, for the removal of chloramines, for 24 hours at 37 ℃. After incubation cell viability was measured using the MTT reduction assay, it was found that there was no significant effect of the removal of chloramines on the toxicity of oxLDL at both 0.5 and 1.0 mg/ml concentrations.

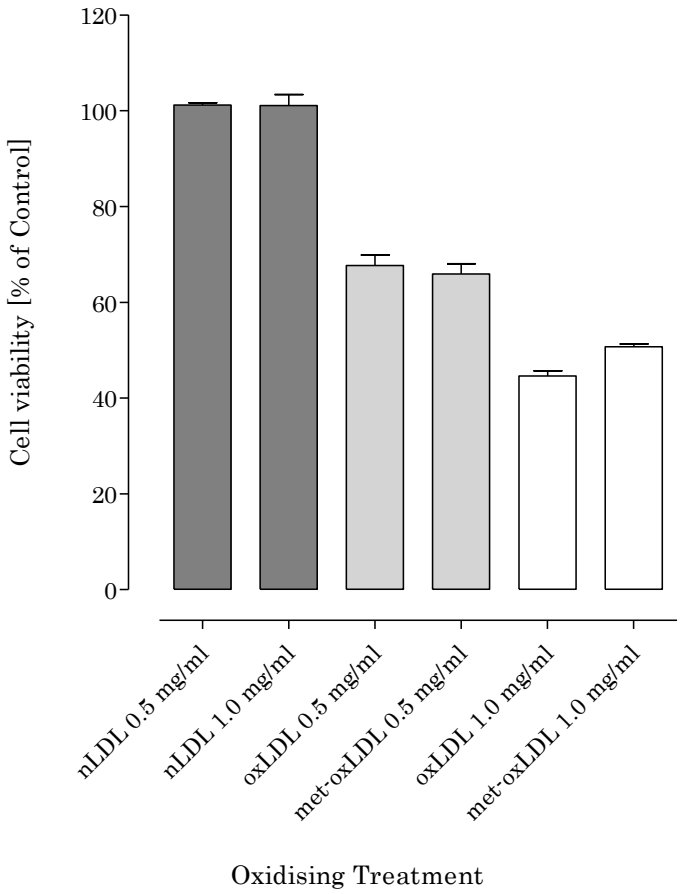


Figure 3.13 Addition of methionine in the oxidation of LDL with HOCl had no effect on cell viability loss in U937 cells upon exposure to HOCl-oxLDL. LDL (2.5mg/ml) was incubated at 37℃ with HOCl for 30 minutes at an oxidant/protein molar ratio of 1000/1. During dialysis treatment, methionine was added in two- fold excess to HOCl to remove chloramines. U937 cells (0.5 x 10^6 cells/ml) in RPMI-1640 were incubated with or without HOCl-oxLDL (0.5 or 1.0 mg/ml) for 24 hours, and then viability analysed by MTT assay. Viability was measured as a percentage of a control containing the same cell treatment with PBS in place of native or oxLDL. Significance is indicated from the respective non-met oxLDL control (^^^ for 0.5 mg/ml *** 1.0 mg/ml).

3.2.3 Intracellular GSH loss on exposure to oxLDL

To help elucidate the toxic mechanism HOCl-oxLDL exhibits towards U937 cells, intracellular oxidative stress production was measured indirectly by quantifying GSH concentration in cells after exposure to HOCl-oxLDL. Incubation of U937 cells with HOCl-oxLDL caused a marked loss of intracellular GSH (figure 3.14c) closely reflecting the pattern of loss that was demonstrated for cell viability (figure 3.14 a&b). Cell viability was measured with both trypan blue exclusion assay and MTT reduction assay, where they showed similar patterns of viability loss in a concentration dependent manner. Although it appeared that either the MTT assay was under estimating, (or trypan blue assay was over estimating) viability across lower concentrations 0.25, and 0.5 mg/ml with differences in viability of approximately -11 and -9% respectively, but both assays found similar viability percentages at the higher concentrations.

GSH loss of approximately 20, 25, 40 and 60% occurred following incubation with 0.25, 0.5, 0.75 and 1.0 mg/ml HOCl-oxLDL respectively. There was almost complete GSH loss (90%) at 1.25 mg/ml HOCl-oxLDL. This suggests that a loss in intracellular GSH more than 50% (of the original cellular concentration) is enough to lose most of its protective ability against oxidative stress and cause a 50% or greater viability loss upon exposure to HOCl-oxLDL.

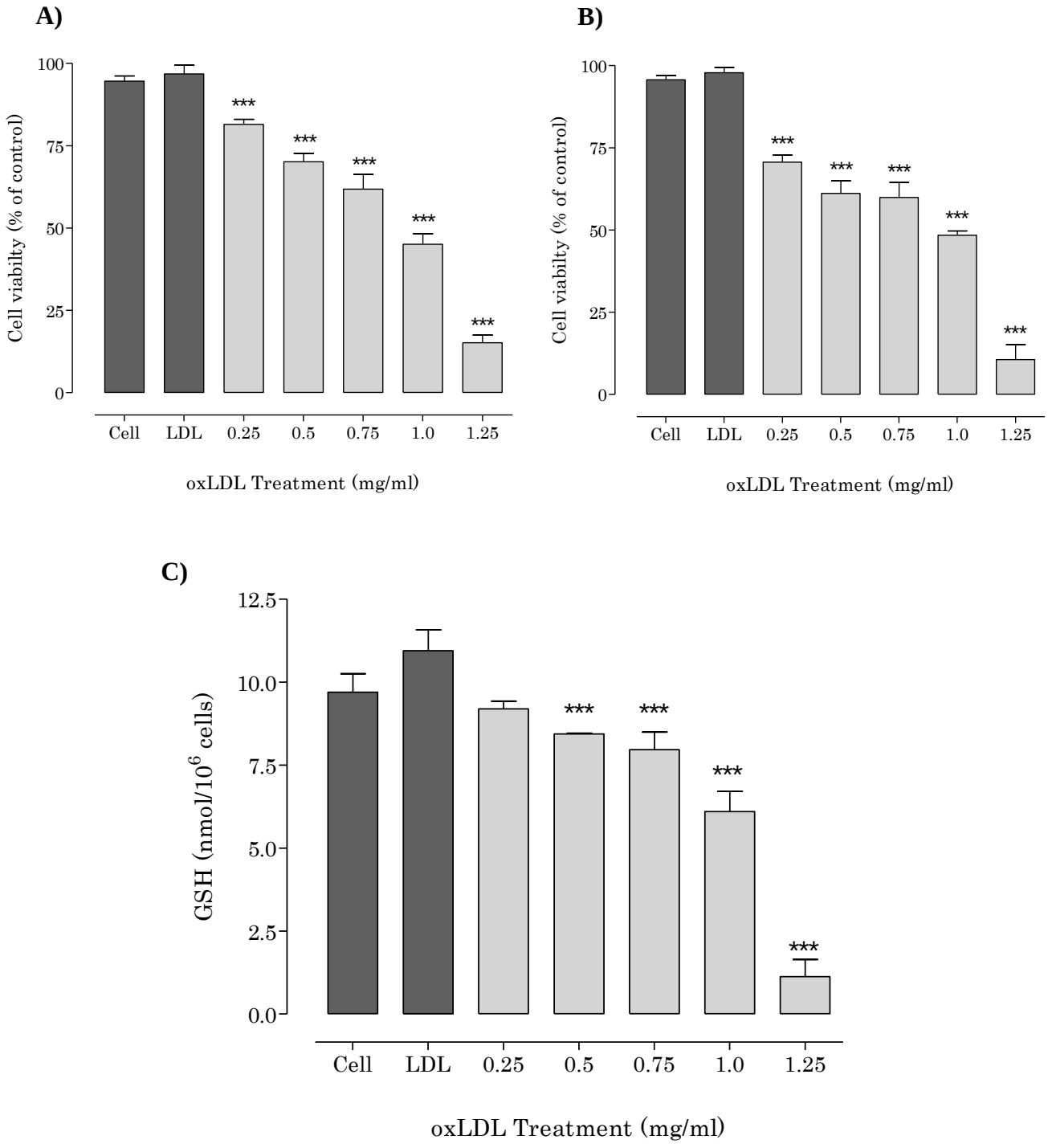


Figure 3.14 Cell viability and intracellular GSH loss in U937 cells upon exposure to varying concentrations of HOCl-oxLDL. Cells (0.5×10^6 cells/ml) were treated with or without varying concentrations of 1000/1 HOCl-oxLDL over 24 hours. After incubation, cells were washed and analysed for viability using the trypan blue exclusion assay **(A)** and the MTT viability assay **(B)**, or analysed for intracellular GSH **(C)** via HPLC analysis. Significance is indicated from the respective 0 mg/ml control.

3.2.4 7,8-Dihydroneopterin protection of U937 cells upon insult of oxLDL

The water-soluble antioxidant compound 7,8-dihydroneopterin (78NP), has been shown by this laboratory, to provide protection to U937 cells against Cu-oxLDL-induced cell death. Specifically, 78NP was shown to prevent Cu-oxLDL-induced intracellular GSH loss by scavenging Cu-oxLDL-induced intracellular oxidants, independently of other antioxidants such as glutathione and α-tocopherol, and therefore maintain the intracellular redox environment (Baird *et al.*, 2004). To further investigate the cytotoxic mechanism of HOCl-oxLDL, and confirm whether oxidative stress was indeed being elevated excessively, as suggested by the previous loss of GSH (figure 3.14), 7,8-dihydroneopterin was utilised as a probe for oxLDL toxicity mechanism. U937 cells were treated with increasing concentrations of 78NP prior to incubation with 1.0 mg/ml oxLDL in the cell medium for 24 hours (figure 3.15). The presence of HOCl-oxLDL without 78NP treatment caused a 54% decrease in viability. Treatment of cells with 50, 100, 200, 250 and 300 μM 78NP before incubation with oxLDL resulted in cell viability loss of only 31, 20, 11, 7 and 5% relative to the cell-only control. This suggests that like Cu-oxLDL, HOCl-oxLDL was generating intracellular oxidants which could be scavenged by the 78NP.

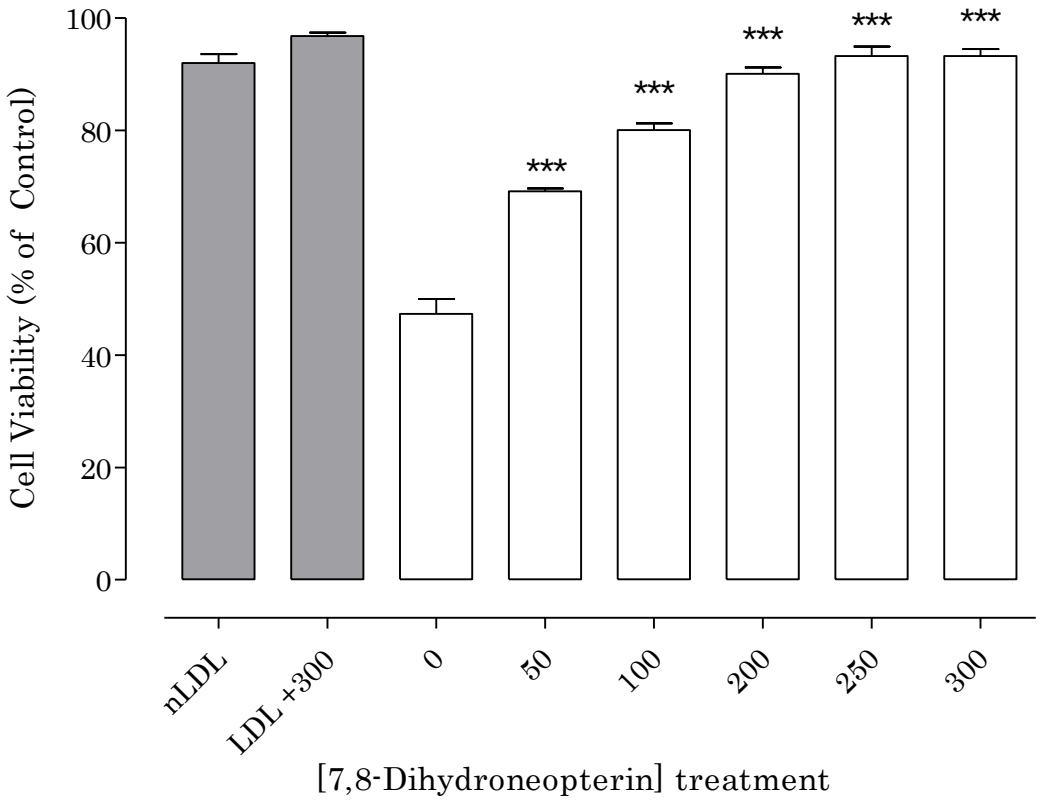


Figure 3.15 7,8-dihydroneopterin (7,8-NP) added to the incubation medium prevented cell viability loss in U937 cells upon exposure to HOCl-oxLDL. Cells (0.5×10^6 cells/ml) were pre-incubated in the presence or absence of varying concentrations of 7,8-NP in the dark at 37°C for 15 minutes, and were subsequently treated with or without 1 mg/ml of 1000/1 HOCl-oxLDL over 24 hours. After incubation cells were washed and analysed for viability using the trypan blue exclusion assay. Viability was measured as ratio of dead versus live cells and expressed as a percentage. Significance is indicated from the 0 µM 78-NP control.

3.2.5 Kinetics of viability and glutathione loss upon insult with oxLDL

Incubation of U937 cells with HOCl-oxLDL caused a marked loss of intracellular glutathione (GSH) (figure 3.16b) closely reflecting the pattern of loss that was demonstrated for cell viability (figure 3.16a). These experiments were performed in both the presence and absence of 78NP to examine its effect on GSH loss. The pattern of HOCl-oxLDL-induced GSH loss was studied over 24 hours using 1.0 mg/ml HOCl-oxLDL. This lethal concentration of HOCl-oxLDL caused a rapid loss of intracellular GSH content to 65% of cellular control within 6 hours of incubation and decreased to less than 40% of the initial intracellular GSH level after 9 hours. The pattern of intracellular GSH loss illustrated here closely mirrors the pattern of cell viability loss (Figure 3.16a).

As with cell viability, the presence of 78NP appeared to dramatically prevent HOCl-oxLDL-induced damage by way of GSH loss, and appeared to have the greatest effect at 9 hours, the presence of 78NP caused a 40% increase in cellular GSH content at this time point compared to HOCl-oxLDL alone, preventing around 30% viability loss.

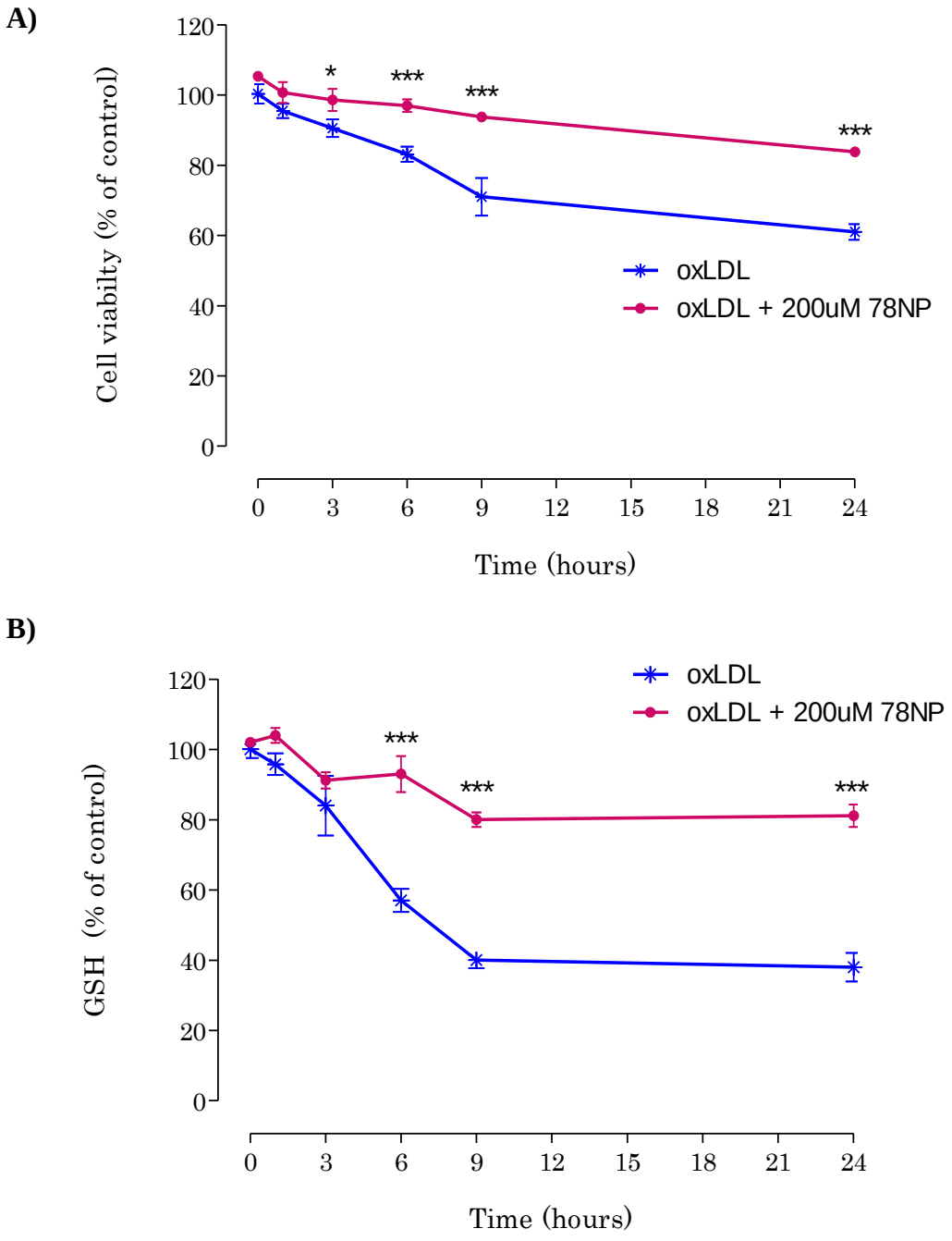


Figure 3.16 7,8-dihydroneopterin (7,8-NP) added to the incubation medium prevented GSH and cell viability losses in U937 cells upon exposure to HOCl-oxLDL. Cells (0.5×10^6 cells/ml) were pre-incubated in the presence or absence of 7,8-NP (200 µM) in the dark at 37°C for 15 minutes, cells were then treated with or without 1 mg/ml 1000/1 HOCl-oxLDL over 24 hours. At set time points samples were taken out, cells were washed and analysed for viability using the MTT viability assay **(A)** [control contained 0.5×10^6 cells/ml in RPMI plus PBS in place of native or oxLDL**],** or analysed for intracellular GSH **(B)** via HPLC analysis [cell control value = 11.2 ± 0.23 Moles GSH/ Moles LDL]. Significance is indicated from the respective 0 hour time point control.

3.2.6 7,8-dihydroneopterin prevented oxidative stress upon exposure to oxLDL

Dihydroethidium (DHE), by virtue of its ability to freely permeate cell membranes is used extensively to monitor superoxide production. DHE upon reaction with superoxide anions forms a red fluorescent product (ethidium) which can be quantified using flow cytometry. To fully confirm the presence of oxidative stress and thus the possible cytotoxic mechanism of HOCl-oxLDL, cells were incubated with HOCl-oxLDL in the presence or absence of 78NP. During incubation cells were washed, probed with DHE and oxidative stress was quantified using flow cytometry. Each sample had 10,000 events recorded within a gate set to exclude cellular debris. Consistent with previous results from this laboratory, mean fluorescence intensity rises rapidly and peaks at 3 hours. This can be observed (figure 3.17a) with the increasing shift to the right along the x-axis as time increases, the widening (and shortening) of the peaks does not suggest that less cells were observed at that time point, rather that more cells within the gated sample showed some form of fluorescence intensity but at a more varying range.

As with cell viability and GSH loss, the presence of 78NP appeared to significantly reduce HOCl-oxLDL-induced damage by preventing oxidative stress (figure 3.17b). It appeared to have the greatest effect at 3 hours, preventing the largest rise in mean fluorescence seen with HOCl-oxLDL alone. It caused near complete retention of normal cell fluorescence as compared to a cell only control across the entire 24 hour incubation.

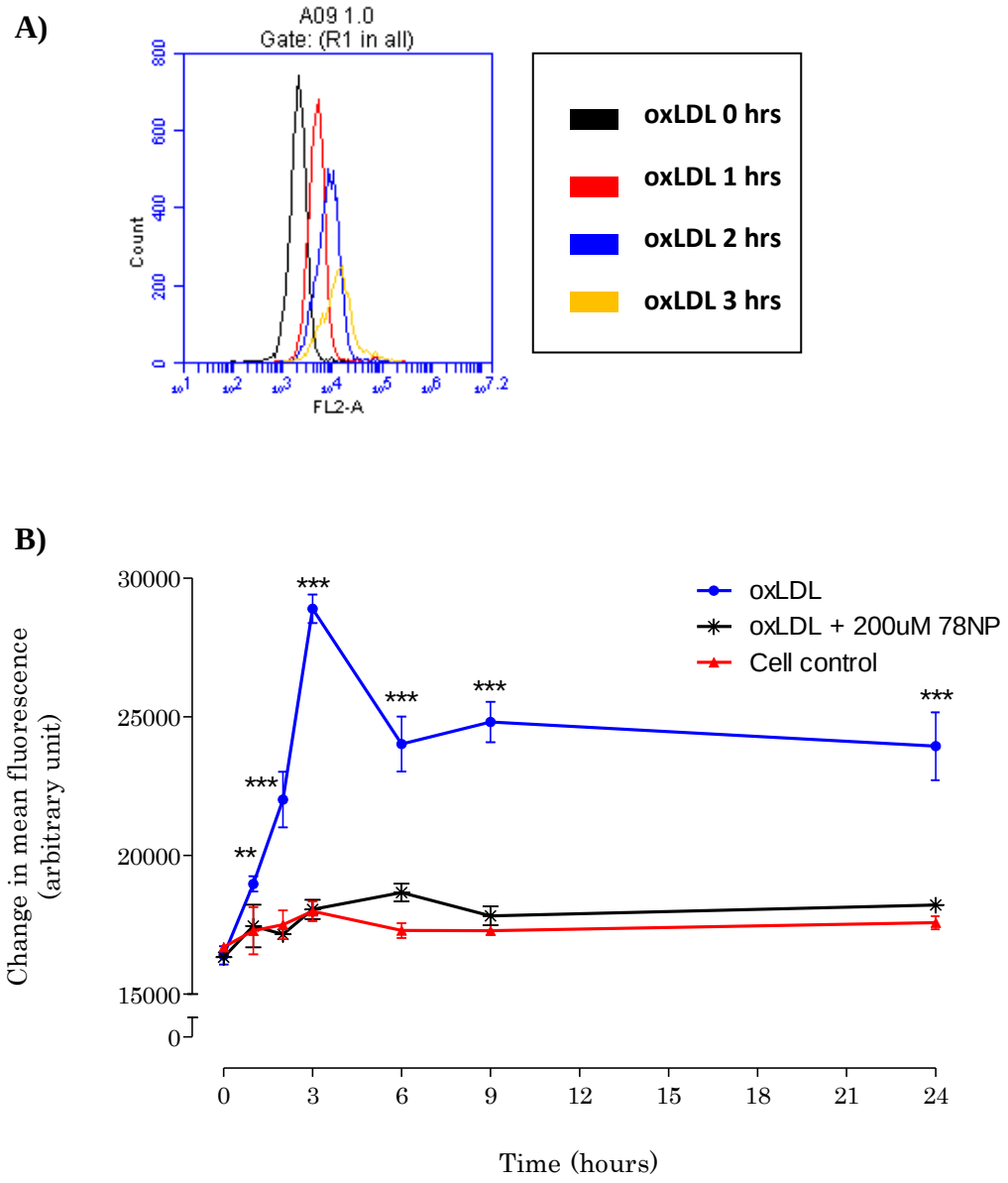


Figure 3.17 7,8-dihydroneopterin (7,8-NP) added to the incubation medium prevented oxidative stress in U937 cells upon exposure to HOCl-oxLDL. Cells (0.5×10^6 cells/ml) were pre-incubated in the presence or absence of 7,8-NP (200 µM) in the dark at 37°C for 15 minutes, then treated with or without 1 mg/ml 1000/1 HOCl-oxLDL over 24 hours. At set time points samples were taken out, and cells analysed for oxidative stress using DHE probe and flow cytometry. Controls consisted of 0.5×10^6 cells/ml, RPMI and PBS in place of HOCl-oxLDL. Significance is indicated between oxLDL and oxLDL + 78NP, at respective time points.

3.3 Characterisation of the modification of Bovine Serum Albumin (BSA) by hypochlorous acid (HOCl)

DNA and lipids are historically considered to be the primary target molecules of free radicals. More recently, proteins have come to light for their role as targets for radical oxidation due to their abundance in biological systems and their ability to bind transition metals (Gebicki *et al.*, 2000).

In contrast to oxidised lipids, the impact of oxidised proteins in atherogenesis has not been extensively studied. Advanced oxidation protein products (AOPPs) are thought to be formed *in vivo* by exposure of serum albumin to HOCl. Significantly increased levels of AOPPs have been found in atherosclerotic lesions, with biochemical characterization revealing that AOPPs are carried by plasma proteins, especially albumin. (Liu *et al.*, 2006).

With the aim of creating a physiologically relevant model for testing the toxicity of AOPPs, pure bovine serum albumin was incubated at 37°C with HOCl at varying oxidant/protein molar ratios to form oxALB. Characterisation of the biochemical properties of oxALB was the first step taken in understanding how HOCl oxidises proteins and how it may play a role in the toxicity of cells within an atherosclerotic inflamation site.

3.3.1 Relative electrophoretic mobility of oxALB

BSA was incubated with varying oxidant/protein molar ratios of HOCl for 30 minutes at 37℃. Relative electrophoretic mobility (REM) of (un)modified BSA was examined to confirm that oxidation had occurred, and to observe how fragmented the oxALB had become. The size of native BSA, and varying oxALB treatments was compared using reducing SDS-PAGE gel electrophoresis (figure 3.18). The albumin band at 66.4kD is significantly modified in the presence of HOCl. As the HOCl concentrations increased the more smeared and fragmented the albumin band becomes. At 2000/1 the oxALB is so fragmented that it becomes difficult to discern individual peptide fragments.

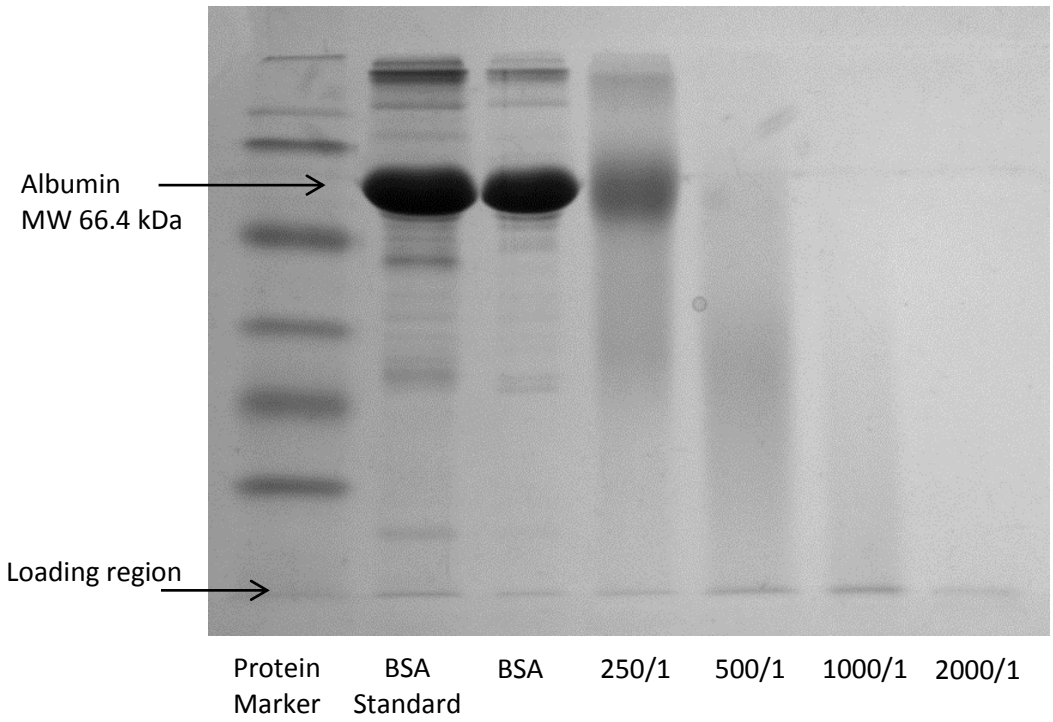
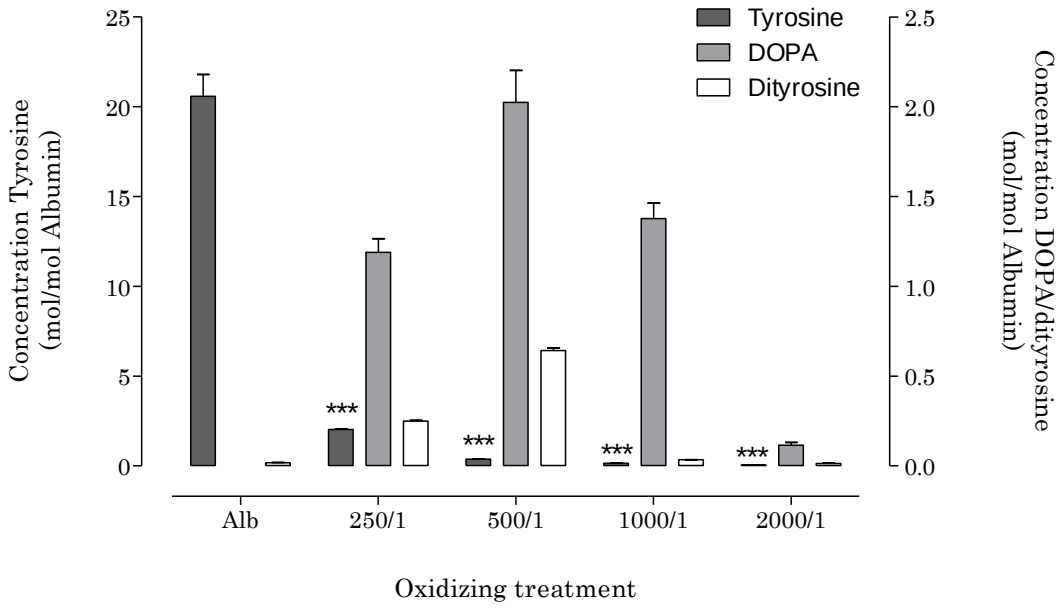


Figure 3.18 SDS-PAGE gel of native and modified BSA. Samples of native and modified BSA (50 µg protein), having undergone lysis buffer treatment, were loaded onto a gradient polyacrylamide gel, along with 5 µl of pre-stained molecular weight marker and run for a 15minutes at 100 V then for a further hour at 200 V.

3.3.2 Tyrosine loss on oxidised BSA

To determine if BSA was indeed oxidised as the SDS-PAGE gel suggested (figure 3.18) tyrosine levels and two of its oxidative products (DOPA and dityrosine) were measured after modification of BSA with HOCl. Protein bound tyrosine, DOPA and dityrosine were quantified though acid hydrolysis and subsequent analysis with HPLC (figure 3.19). Significant reductions in the levels of tyrosine were observed with increasing molar excesses. At the lowest molar ratio (250/1) 90% of native levels were lost, with complete loss of tyrosine detected at 1000/1 and 2000/1. There was a significant ($P < 0.05$) rise in DOPA from native levels to 250/1 and 500/1 treatments, then it dropped off again in 1000/1 and was almost undetectable at 2000/1. There was a rise in the level of dityrosine detected at 250/1 and 500/1, however, these were statistically insignificant.



3.19 Concentration of tyrosine and resulting increases to tyrosine oxidation products (DOPA and dityrosine) after oxidation of BSA with HOCl. BSA (7.5 mg/ml) was oxidised following incubation with HOCl at 37℃ for 30 minutes at concentrations corresponding to oxidant/protein molar ratios of 250/1, 500/1, 1000/1 and 2000/1. Oxidised BSA was diluted 1:5 with nanopure water to give a final concentration of 1 mg/ml before acid hydrolysis. The concentration of tyrosine and its oxidative products were determined through HPLC analysis of the acid hydrolysate. Significance is indicated from the respective native BSA control.

3.3.3 PrOOH formation in BSA after exposure to HOCl

Proteins damaged by free-radical-generating systems in the presence of oxygen yield relatively long-lived protein hydroperoxides (PrOOHs). PrOOHs are also formed on amino acids and proteins during oxidative stress, and have the potential to cause further biological damage. PrOOH formation on the samples analysed immediately after oxidation (figure 3.20) increased in an almost linear manner up to the 1000/1 treatment where the concentration peaked at 0.27 moles PrOOH/moles BSA, before levels declined in subsequent higher molar ratio treatments.

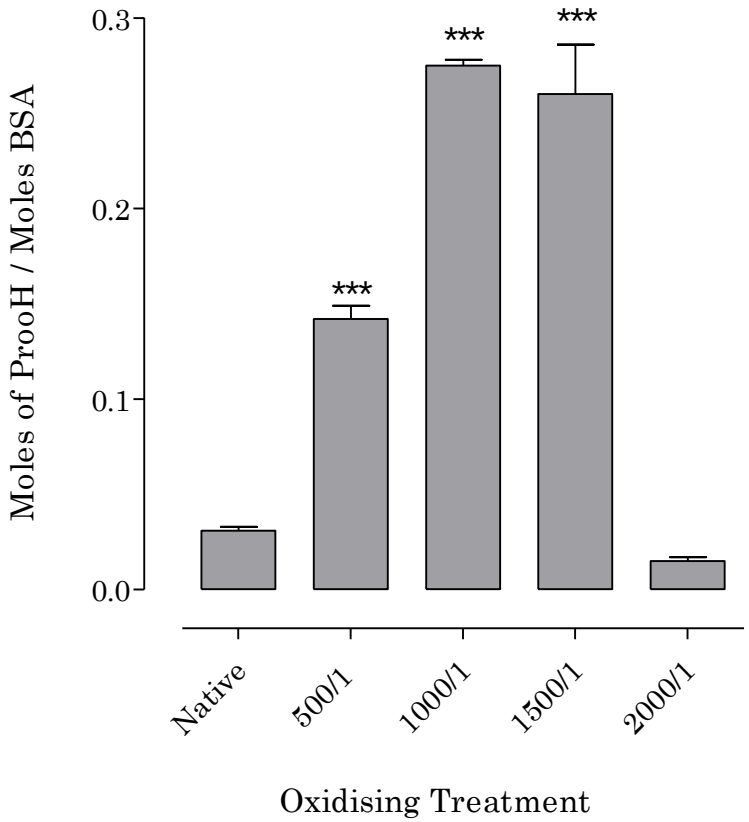


Figure 3.20 PrOOH formation on albumin when oxidised by varying HOCl treatments. BSA (7.5 mg/ml) was incubated at 37℃ with HOCl for 30 minutes. Samples were removed immediately after oxidation and analysed for protein hydroperoxides using the acetic acid-FOX assay. PrOOHs were adjusted against blank controls containing FOX reagents and 50% acetic acid. Significance is indicated from the native BSA control.

3.4 Toxicity of oxALB

Despite finding elevated levels of oxidised proteins in a wide range of diseases, including atherosclerosis, their possible role in the progression of these diseases has not been elucidated. It has been postulated that these AOPPS are in fact toxic to cells but no conclusive evidence has been brought to light showing evidence of this *in vivo*. Aside from assessing whether oxALB displays cytotoxic properties, the next set of experiments aimed to establish appropriate concentrations of oxALB at the best oxidant/protein molar ratio, for use in later experiments.

3.4.1 Cytotoxicity of oxALB on U937 cells

After oxidation it was noted that oxALB had a strong pungent smell to it, reminiscent of a commercial swimming pool, it was suggested that this smell could be coming from a cache of unreacted HOCl within the oxALB sample. To eliminate the fact that HOCl could be a contributing factor in the toxicity of oxALB, residual HOCl was removed after oxidation though a 3 step dialysis against PBS.

The toxicity of oxALB to U937 cells was examined by exposing U937 cells to increasing oxidant/protein molar ratios of oxALB in RPMI-1640 cell medium for 24 hours. Cell viability was subsequently measured using the MTT reduction assay (figure 3.21). In contrast to the cytotoxic effects of oxLDL (figure 3.11), oxALB displayed statistically significant differences between increasing oxidant/protein ratios and their effect on cell viability. This was especially emphasised on oxALB that had undergone dialysis treatment (figure 3.21b).

Cells exposed to oxALB that had not been dialysed (figure 3.21a) had their viability reduced dramatically down to 15% at the lowest oxidant/protein molar ratio through to 7% of the cell only control at 2000/1. Compared to oxALB that had been dialysed (figure 3.21b) there was a marked increase in viability with rises of 73, 69, and 39% at 250/1, 500/1 and 1000/1 respectively.

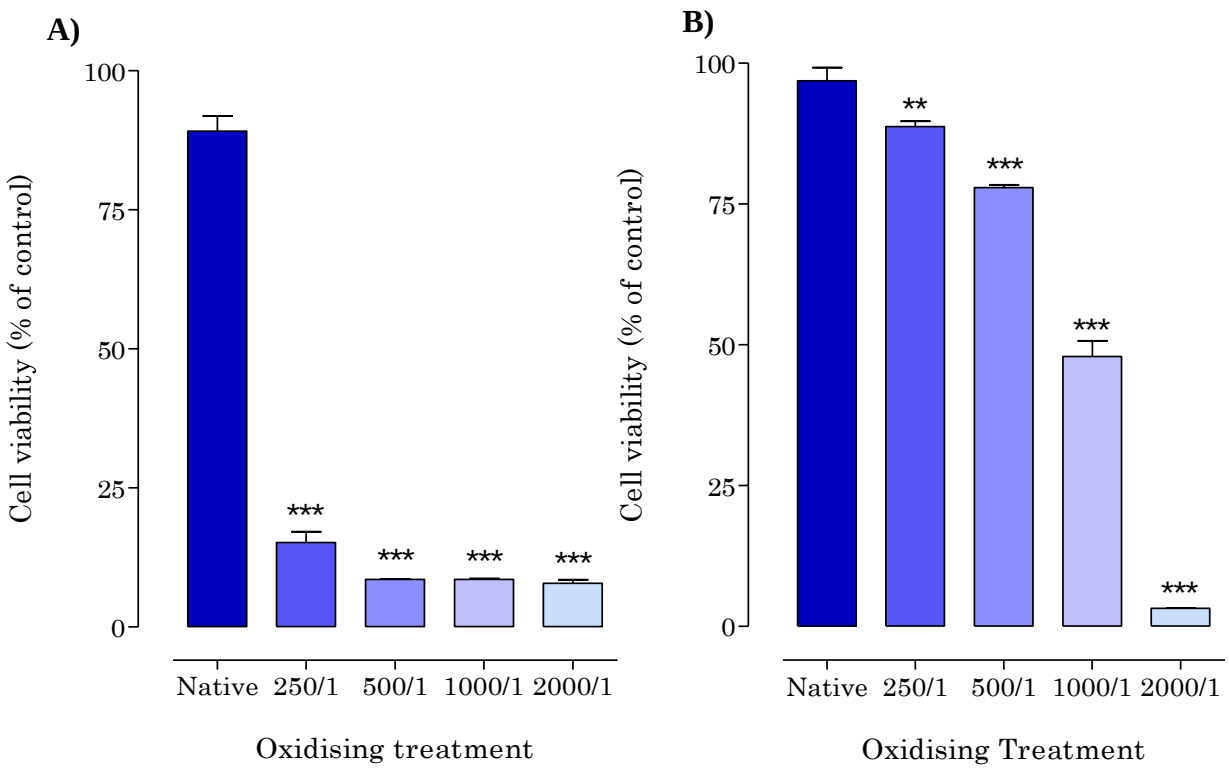


Figure 3.21 Dialysis treatment of oxALB decreased cell viability loss in U937 cells upon exposure to oxALB. BSA (7.5 mg/ml) was incubated at 37℃ with HOCl for 30 minutes at an oxidant/protein molar ratio of 1000/1. OxALB then either did **(B)** or did not **(A)** undergo dialysis to remove any excess HOCl. Cells (0.5 x 10^6 cells/ml) in RPMI-1640 were incubated with or without oxLDL (1.0 mg/ml) for 24 hours, and then viability analysed by MTT assay. Viability was measured as a percentage of a control containing the same cell treatment with PBS in place of native or oxALB. Significance is indicated from the respective native BSA control.

The difference between the two treatments (dialysed versus non-dialysed) was most likely due to residual HOCl in the oxALB solution. After establishing that increasing oxidant/protein ratios cause an increase in toxicity, the question arises does cytotoxicity of oxALB have a concentration-dependent effect on viability? This was examined by exposing U937 cells to varying concentrations of 1000/1 oxALB in RPMI-1640 cell medium for 24 hours (figure 3.22). Cell viability was subsequently measured using the MTT reduction assay (figure 3.21). U937 cells displayed a concentration-dependent decrease in cell viability following incubation with oxALB. Comparatively, there was no statistically significant difference between dialysed and non-dialysed samples, the exception being 1.0 mg/ml in which there was a 32% increase in viability.

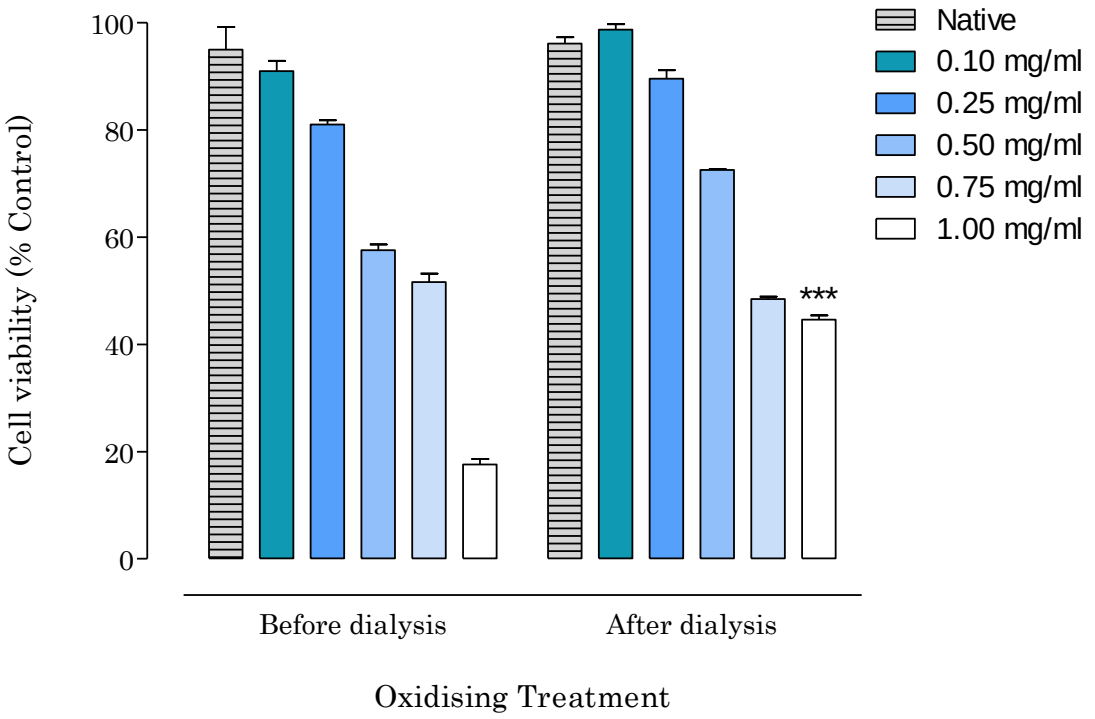


Figure 3.22 Concentration dependent toxicity through exposure of dialysed and non-dialysed oxALB to U937 cells. BSA (7.5 mg/ml) was incubated at 37℃ with HOCl for 30 minutes at an oxidant/protein molar ratio of 1000/1. OxALB then either did **(**or did not) undergo dialysis to remove any excess HOCl. U937 cells (0.5 x 10^6 cells/ml) in RPMI-1640 were incubated with varying concentrations of oxALB for 24 hours, and then viability analysed by MTT assay. Viability was measured as a percentage of a control containing the same cell treatment with PBS in place of native or oxALB. Significance is indicated between the respective before and after samples.

The precise toxicity of oxALB to U937 cells was found to vary slightly between different batches prepared, with the median lethal dose (LD_{50}) between 0.6 -1.0 mg/ml oxALB. The most consistent LD_{50} occurred at 1000/1 molar ratio with oxALB that had been dialysed, and so it was decided that subsequent experiments would be conducted at 1.0mg/ml with the 1000/1 treatment, unless otherwise stated.

3.4.2 Comparison of cytotoxicity between HOCl-oxLDL and oxALB on U937 cells

The toxicity of oxALB and HOCl-oxLDL to U937 cells was examined by incubating cells with both oxidants at equivalent total mass (1.0 mg/ml) for 24 hours. Cell viability was subsequently measured using the MTT reduction assay (figure 3.23). Comparatively, HOCl-oxLDL was more toxic than oxALB at the two lowest oxidant/protein molar ratios (250/1 and 500/1), at the 1000/1 ratio there was no significant difference between the level of toxicity, and at the highest ratio oxALB was markedly more toxic with only 3% cell viability after treatment compared to 37% with HOCl-oxLDL. Contrasting to the cytotoxic effects of HOCl-oxLDL, oxALB displayed statistically significant differences between increasing oxidant/protein ratios and their effect on cell viability.

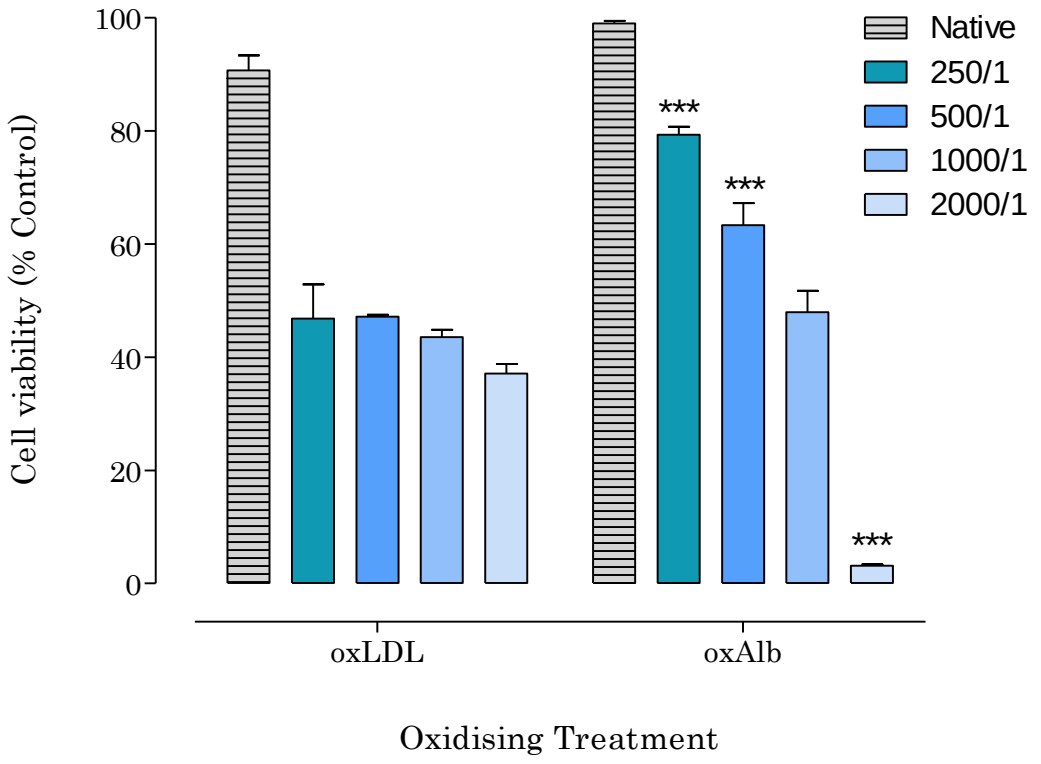


Figure 3.23 Comparison of HOCl-oxLDL and oxidised albumin with varying HOCl treatments given to U937 cells at equivalent total mass (1 mg/ml). LDL and Albumin were incubated at 37℃ with HOCl for 30 minutes at concentrations corresponding to oxidant/protein molar ratios of 250/1, 500/1, 1000/1, and 2000/1 to form HOCl-oxLDL and oxALB. U937 cells (0.5×10^6 cells/ml) in RPMI-1640 were incubated with 1.0 mg/ml oxLDL or 1.0 mg/ml oxALB for 24 hours, and analysed by MTT assay. Viability was measured as a percentage of a control containing the same cell treatment with PBS in place of oxidative solution. Significance is indicated between the respective oxLDL and oxALB treatments.

To assess whether the toxicity both HOCl-oxLDL and oxALB was due to some toxic component produced from the protein moiety or a derivative of protein oxidation, we measured comparative toxicity at equivalent protein mass. The toxicity was examined by incubating cells with either HOCl-oxLDL at 1.0 mg/ml or oxALB at 0.2 mg/ml, for 24 hours. Thus the cells were exposed to either 0.2 mg/ml apoB-100 or 0.2 mg/ml oxALB. Cell viability was subsequently measured using the MTT reduction assay (figure 3.24). OxALB toxicity was dramatically reduced compared to oxLDL across all oxidant/protein molar ratios, with increases in viability of 47, 45, 46 and 25% compared to the respective oxLDL treatments.

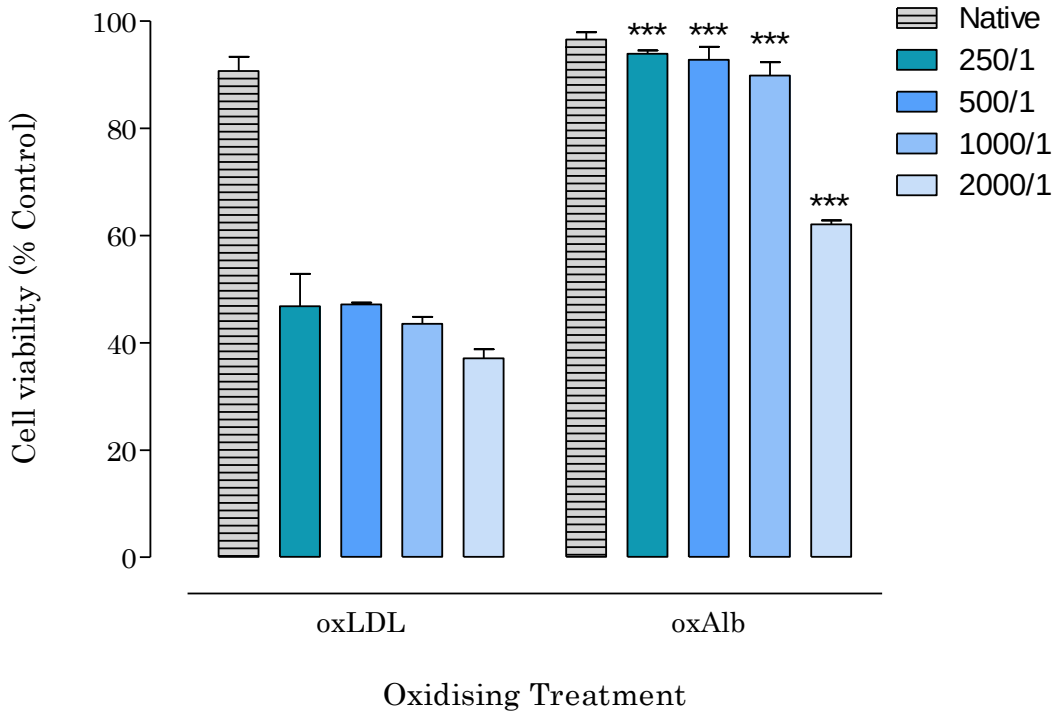


Figure 3.24 Comparison of HOCl-oxLDL and oxidised albumin with varying HOCl treatments given to U937 cells at equivalent protein mass. LDL and Albumin were incubated at 37℃ with HOCl for 30 minutes at concentrations corresponding to oxidant/protein molar ratios of 250/1, 500/1, 1000/1, and 2000/1 to form HOCl-oxLDL and oxALB. U937 cells (0.5 x 10^6cells/ml) in RPMI-1640 were incubated with 1.0 mg/ml HOCl-oxLDL (containing 0.2 mg/ml ApoB-100) or 0.2 mg/ml oxALB for 24 hours, and analysed by MTT assay. Viability was measured as a percentage of a control containing the same cell treatment with PBS in place of oxidative solution. Significance is indicated between the respective oxLDL and oxALB treatments.

3.4.3 Presence of foetal bovine serum shows minimal protection against the cytotoxicity of oxALB

It has been shown previously in this laboratory that the presence of foetal bovine serum (FBS) can have a protective effect against oxidative stress due to exposure of Cu-oxLDL. Could FBS also protect cells against the cytotoxicity of oxALB? This was assessed by incubating cells with oxALB at 1.0 mg/ml in the presence and absence of 5% FBS, after experimental treatment, cell viability was measured using the MTT viability assay (figure 3.25). Consequently it was found that presence of FBS had little to no effect on the toxicity of oxALB with only slightly significant ($P < 0.01$) increases in viability of 9% and 7% at the 500/1 and 2000/1 ratios respectively.

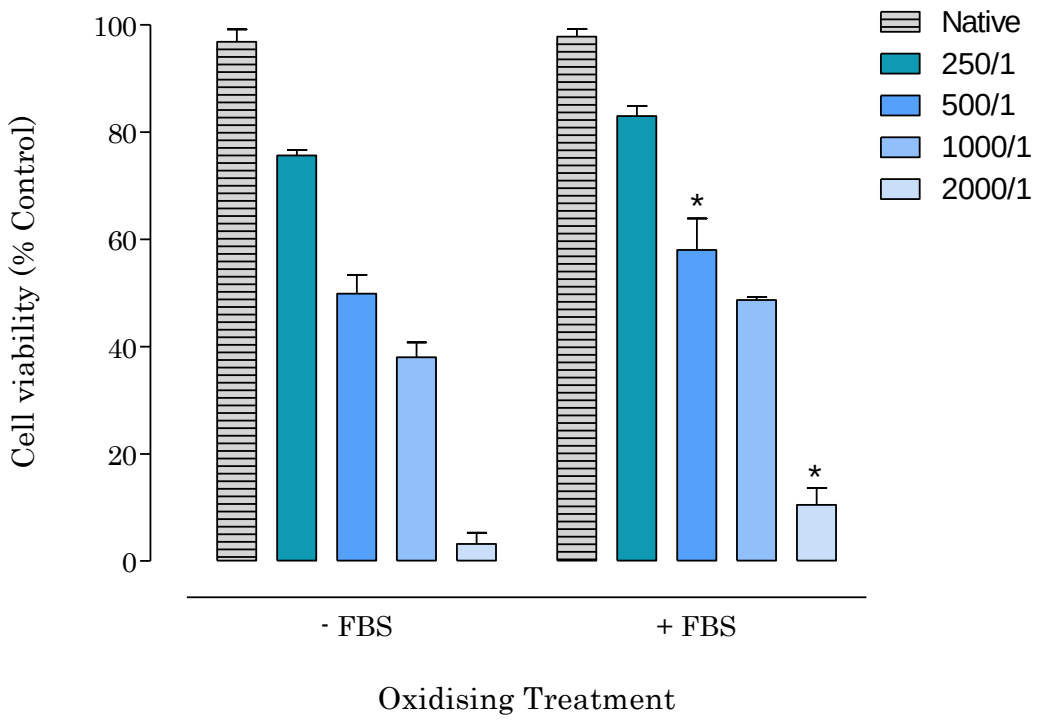


Figure 3.25 Toxicity of oxALB in the presence of FBS on U937 cells after 24 hours. Albumin (7.5 mg/ml) was incubated at 37℃ with HOCl for 30 minutes at concentrations corresponding to oxidant/protein molar ratios of 250/1, 500/1, 1000/1, and 2000/1 to form oxALB. U937 cells (0.5 x 10^6 cells/ml) in RPMI-1640 were incubated in the absence **(A),** or presence **(B)** of foetal bovine serum, with 1.0 mg/ml oxALB for 24 hours, and the results analysed by MTT assay. Viability was measured as a percentage of a control containing the same cell treatment with PBS in place of oxALB. Significance is indicated between the respective absence and presence of FBS treatments.

3.4.4 Effect of chloramines on the toxicity of oxALB

Free amino acids readily form chloramines in reactions with HOCl, however the reaction can be postponed by the presence of amino acids whose side groups have reducing properties such as methionine (Naskalski & Bartosz 2001).

The potential chloramines were removed from the oxALB during the dialysis step using excess methionine (figure 3.26). Cells were incubated with or without oxALB (0.5 and 1.0 mg/ml) that had either been treated (or not treated) for the removal of chloramines, for 24 hours at 37℃. After experimental treatment cells were checked for viability using the MTT reduction assay, where it was found that there were statistically significant effects of the removal of chloramines on the toxicity of oxALB at both 0.5 and 1.0 mg/ml concentrations.

There appears to be some biphasic mechanism of toxicity. Although removal of chloramines did increase viability by 46% and 22% at 0.5 and 1.0 mg/ml respectively, methionine-treated oxALB still caused cell viability loss, especially in the 1.0 mg.ml treatment where viability was reduced by 75%.

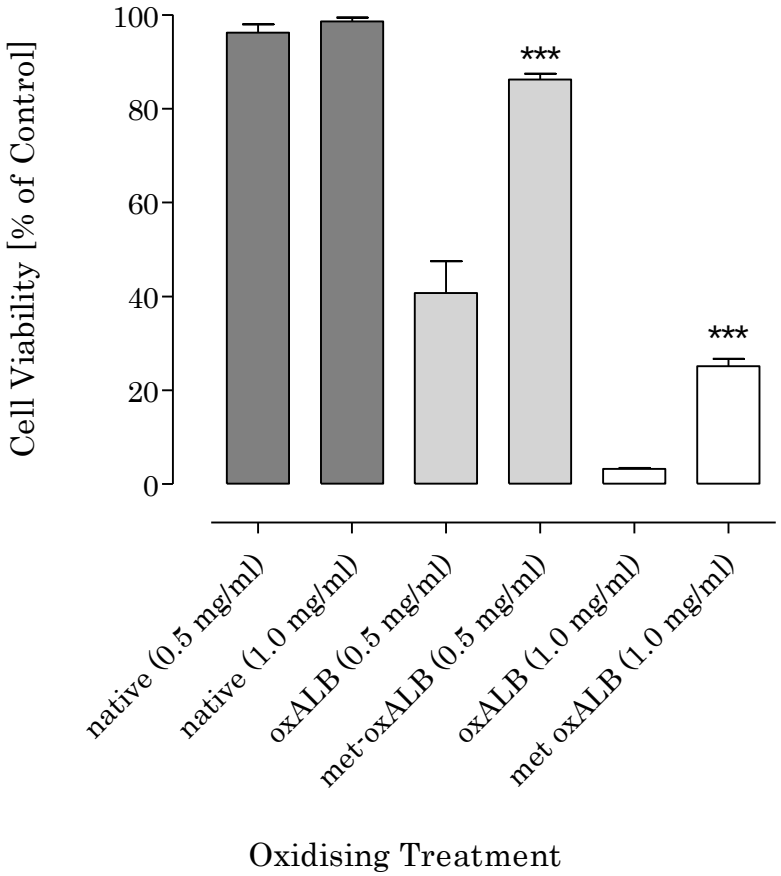


Figure 3.26 Addition of methionine in the oxidation of BSA and its effect on cell viability loss in U937 cells upon exposure to oxALB. BSA (7.5 mg/ml) was incubated at 37℃ with HOCl for 30 minutes at an oxidant/protein molar ratio of 1000/1. During dialysis treatment, methionine was added in two- fold excess to HOCl to remove chloramines. U937 cells (0.5 x 10^6 cells/ml) in RPMI-1640 were incubated with or without oxALB (0.5 or 1.0 mg/ml) for 24 hours. Viability was analysed by MTT assay and measured as a percentage of a control containing the same cell treatment with PBS in place of native or oxLDL. Significance is indicated from the respective native BSA controls (0.5 or 1.0 mg/ml).

To assess whether chloramines played a role in the differing of toxicity between oxidant/protein molar ratios, chloramines were removed during the dialysis step using excess methionine (figure 3.27). Cells were incubated with or without oxALB at varying ratios that had either been treated (or not treated) for the removal of chloramines and subsequently tested for cell viability using the MTT reduction assay. There were statistically significant effects from the removal of chloramines on the toxicity of oxALB at the 1000/1 and 2000/1 ratios which increased viability by 17 and 24% respectively.

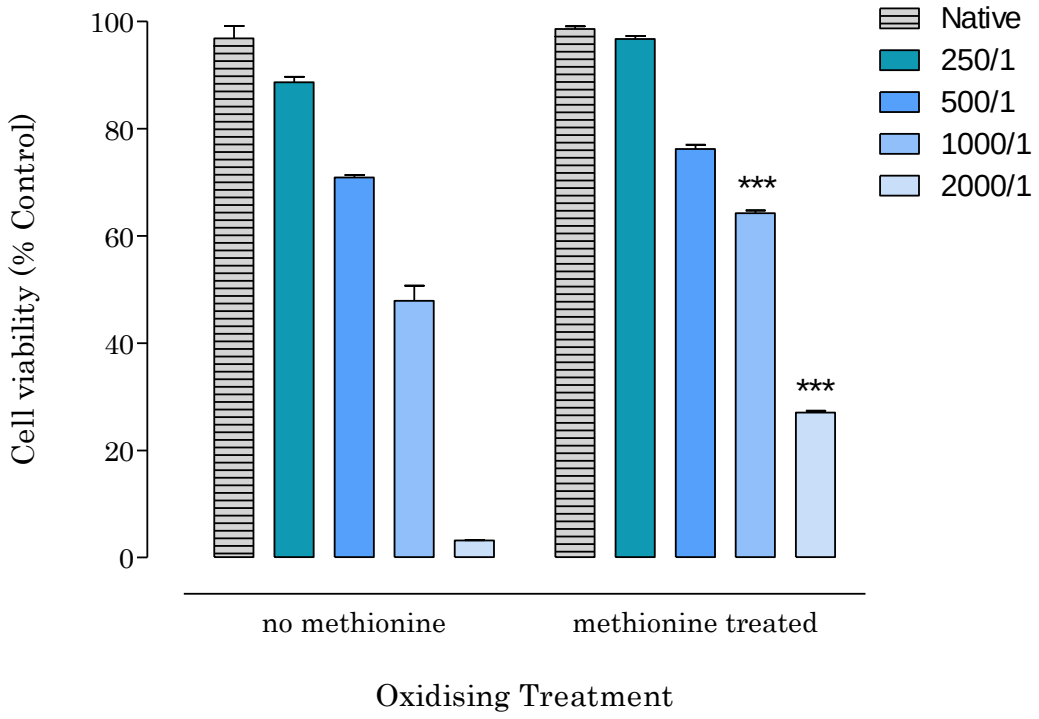


Figure 3.27 Addition of methionine in the oxidation of BSA and its effect on cell viability loss in U937 cells upon exposure to oxALB. BSA (7.5 mg/ml) was incubated at 37℃ with HOCl for 30 minutes at concentrations corresponding to oxidant/protein molar ratios of 250/1, 500/1, 1000/1, and 2000/1 to form oxALB. During dialysis treatment, methionine was either added (or not) in two- fold excess to HOCl to remove chloramines. U937 cells (0.5 x 10^6 cells/ml) in RPMI-1640 were incubated with or without oxALB (1.0 mg/ml) for 24 hours, and then viability analysed by MTT assay. Viability was measured as a percentage of a control containing the same cell treatment with PBS in place of native or oxLDL. Significance is indicated between the respective non-methionine and methionine treated samples.

Time course studies of oxALB incubated with U937 cells provided additional evidence for the role chloramines play in the toxicity of oxALB, by assessing if their removal had any effect on the rate of cell death. A time course study of oxALB cytotoxicity over 24 hours with 1.0 mg/ml oxALB was performed. At set time points samples were analysed for viability using both the MTT and trypan blue assays (figure 3.28a). In a parallel experiment chloramines were removed during dialysis and subsequently viability was measured (figure 3.28b). These experiments answered several points of interest; one, that there was no significant difference between the two viability assays (confirming the validity of the MTT assay which has been questioned in the past for over estimating viability loss) and two, further confirming the presence of chloramines and their role in the cytotoxicity of oxALB.

The results illustrated a rapid decrease in viability that occurred primarily within the first 6 hours for non-methionine treated oxALB (figure 3.28a). Although marked viability loss in methionine treated oxLDL treatment did occur, it had an initial lag phase before dropping off suddenly to have most loss occurring between 6 and 9 hours (figure 3.28b).The removal of chloramines did show a significant increase in viability with maximum loss reaching around 84% compared to non-methionine treatment which achieved a 97% loss in cell viability compared to cell only control.

The use of trypan blue assay illuminated the morphology changes that the cells went through upon exposure to oxALB and subsequent cell death, the corresponding microscopy showed that a large degree of cell lysis occurred between 3 and 9 hours in both experiments.

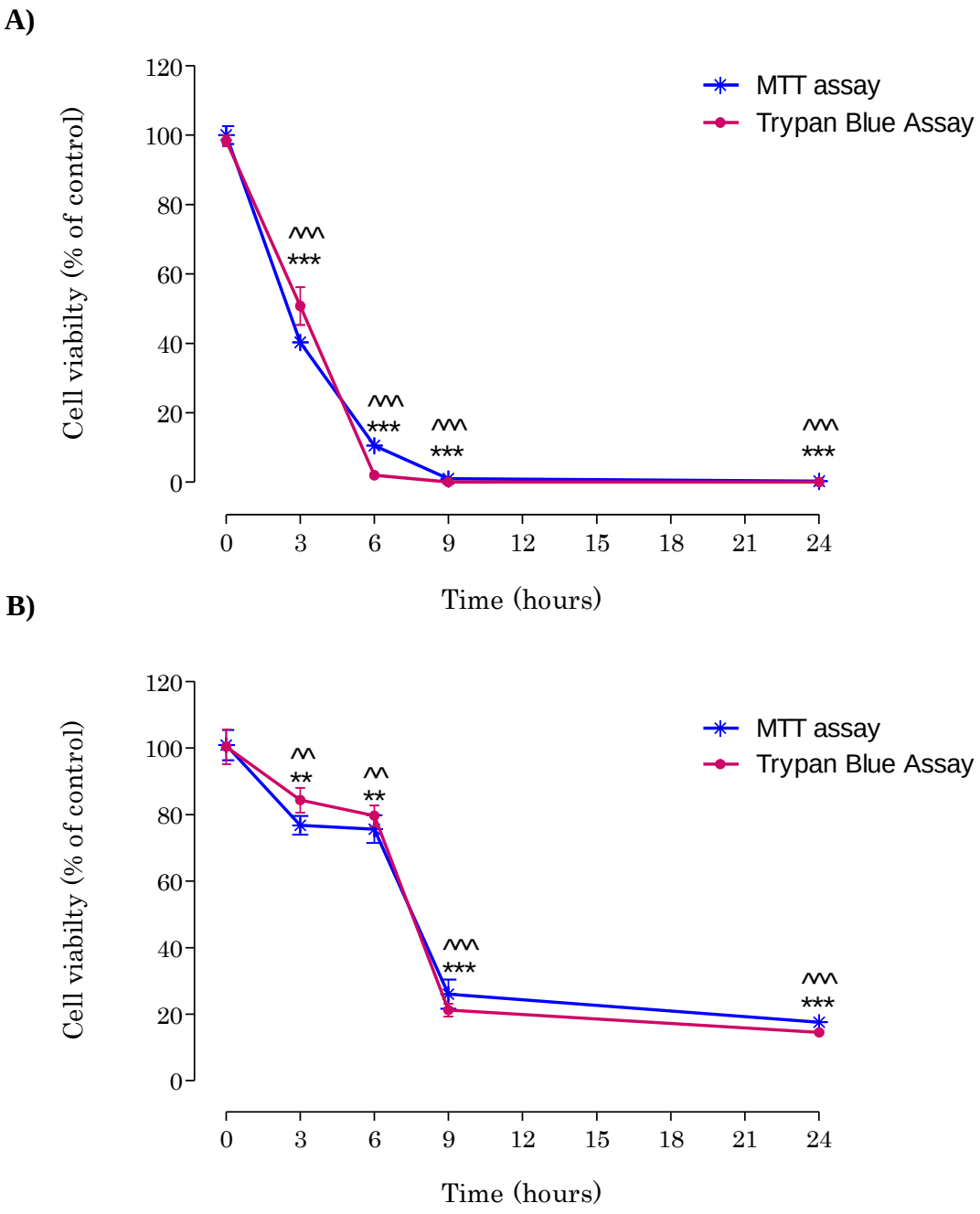


Figure 3.28 Addition of methionine in the oxidation of BSA and its effect on cell viability loss in U937 cells upon exposure to oxALB during a 24 hour time course. BSA (7.5mg/ml) was incubated at 37℃ with HOCl for 30 minutes at an oxidant/protein molar ratio of 1000/1. During dialysis treatment, methionine was either added **(B)** (or not **(A)**) in two-fold excess to HOCl to remove chloramines. U937 cells (0.5 x 10^6 cells/ml) in RPMI-1640 were incubated with or without oxALB (1.0 mg/ml) for 24 hours. At set time points during incubation, samples were taken out, and analysed for viability by the MTT assay and trypan blue exclusion assay. Viability was measured as a percentage of a control containing the same cell treatment with PBS in place of native or oxLDL. Significance is indicated from the respective 0 hour time point control.

3.4.5 Intracellular GSH loss on exposure to oxALB

Although chloramines were found to contribute to the toxicity of oxALB, their removal did not prevent cell death in U937 cells. Cu-oxLDL and HOCl-oxLDL appear to kill U937 cells by inducing an intracellular oxidative stress. It was investigated whether oxALB also caused an oxidative stress in U937 cells. This was assessed by measuring intracellular GSH loss. Incubation of U937 cells with increasing concentrations of oxALB caused a marked loss of intracellular GSH (figure 3.29a). U937 cells displayed a concentration-dependent decrease in GSH loss following incubation with oxALB. Comparatively greater GSH loss occurred in oxALB that had not had chloramines removed, with significant increases in GSH levels found in methionine treated oxALB of 4.1, 4.4, 3.7 and 3.1 nmol GSH/10^6 cells at 0.5, 0.75, 1.0 and 1.25 mg/ml treatments respectively.

There were differences between increasing oxidant/protein molar ratios and their effect on intracellular GSH loss (figure 3.29b). Cells were incubated with 1.0 mg/ml oxALB (methionine treated) at varying ratios for 24 hours, following which intracellular GSH levels were measured using HPLC. Significant GSH losses of 2.9 and 10.6 nmol GSH/10^6cells were seen at 500/1 and 1000/1 ratios compared to cell only control. However at 250/1 there was actually a small but statistically insignificant increase in GSH levels possibly suggesting the cells in the lower HOCl/BSA molar ratio treatment triggered some sort of survival mechanism increasing cellular GSH content.

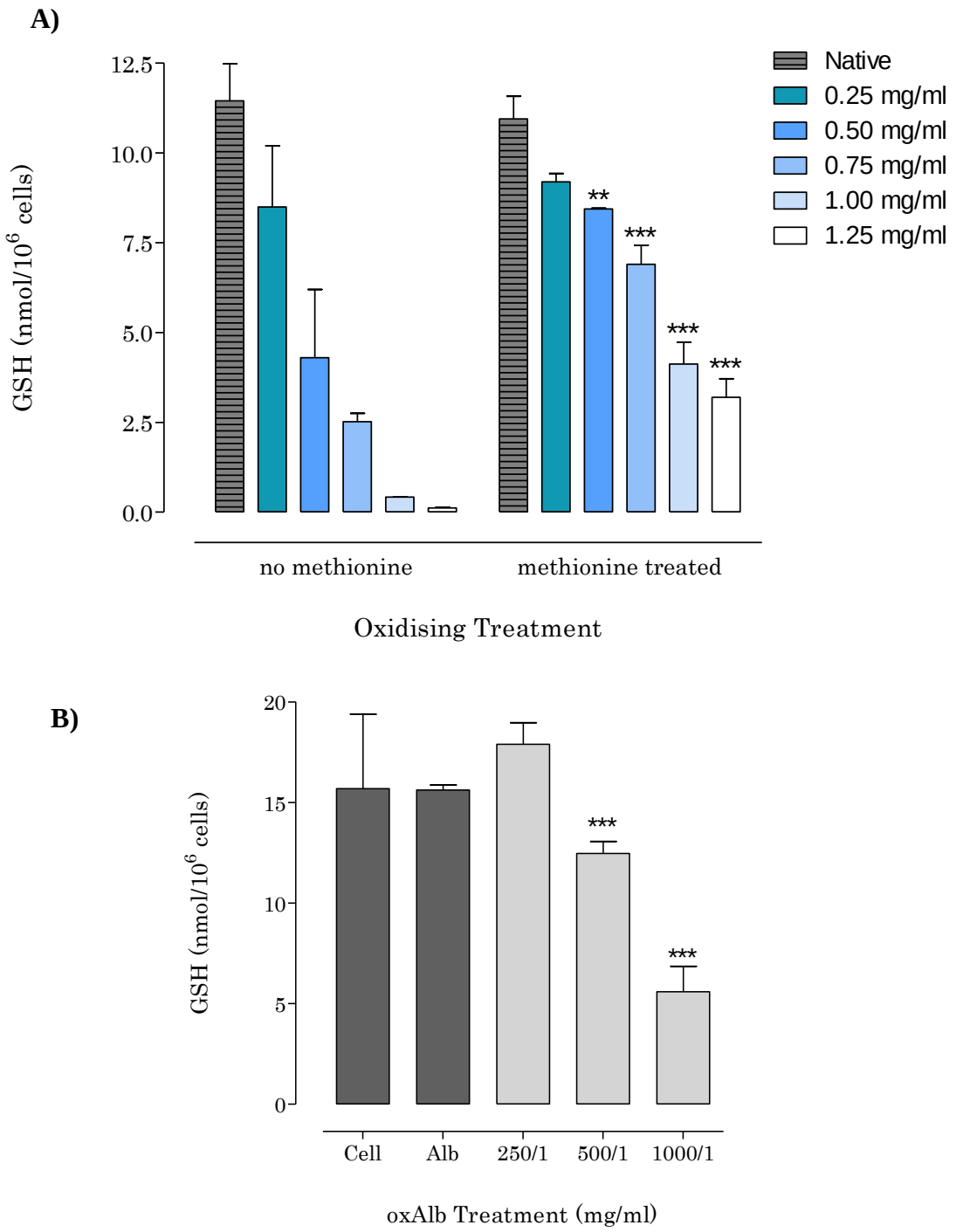


Figure 3.29 Addition of methionine in the oxidation of BSA and its effect on intracellular GSH loss in U937 cells upon exposure to oxALB. Cells (0.5 x 10^6 cells/ml) in RPMI-1640 were incubated with or without 1000/1 oxALB (treated with or without methionine), at varying concentrations **(A)**, or with varying oxidant/protein ratios of met-oxALB at 1.0 mg/ml **(B)**, for 24 hours and then analysed for intracellular GSH via HPLC analysis. Significance is indicated from the respective 0 mg/ml control **(A)**, or from the native BSA control **(B)**.

3.4.6 7,8-Dihydroneopterin protection of U937 cells upon insult of oxALB

It has been shown previously that 7,8-dihydroneopterin (78NP) inhibits cytotoxicity caused by Cu-oxLDL and now HOCl-oxLDL (see section 3.2); so it was investigated whether it could it also protect against cell death caused by oxALB. As with previous studies suggesting a role for reactive oxygen species (ROS) in cell death, this experiment aimed to determine whether ROS represent a contributing factor in oxALB-induced cell death. U937 cells were treated with increasing concentrations of 78NP prior to incubation with 1.0 mg/ml oxALB (figure 3.30a) or methionine-treated oxALB (figure 3.30b) in the cell medium for 24 hours.

The presence of oxALB without 78NP treatment caused a near complete loss in cellular viability (figure 3.30a). Treatment of cells with 50, 100, 200, 250 and 300 µM 78NP before incubation with oxALB resulted in cell viability loss of only 98, 86, 68, 57, and 45% relative to the cell-only control.

There was much greater protection with 78NP in cells that were exposed to met-oxALB (treated for the removal of chloramines) (figure 3.30b). Although methionine treated oxALB was not as toxic (causing around 82% viability loss compared to untreated oxALB which caused 99.5% loss) the levels in which 78NP raised viability was much greater. Treatment of cells with 50 and 100 µM 78NP before incubation with oxALB resulted in cell viability loss of only 51, 35% relative to the cell-only control. Treatment with 200, 250 and 350 µM 78NP actually raised viability past the control, not necessarily suggesting that these treatments had more alive cells but rather these cells were more metabolically active.

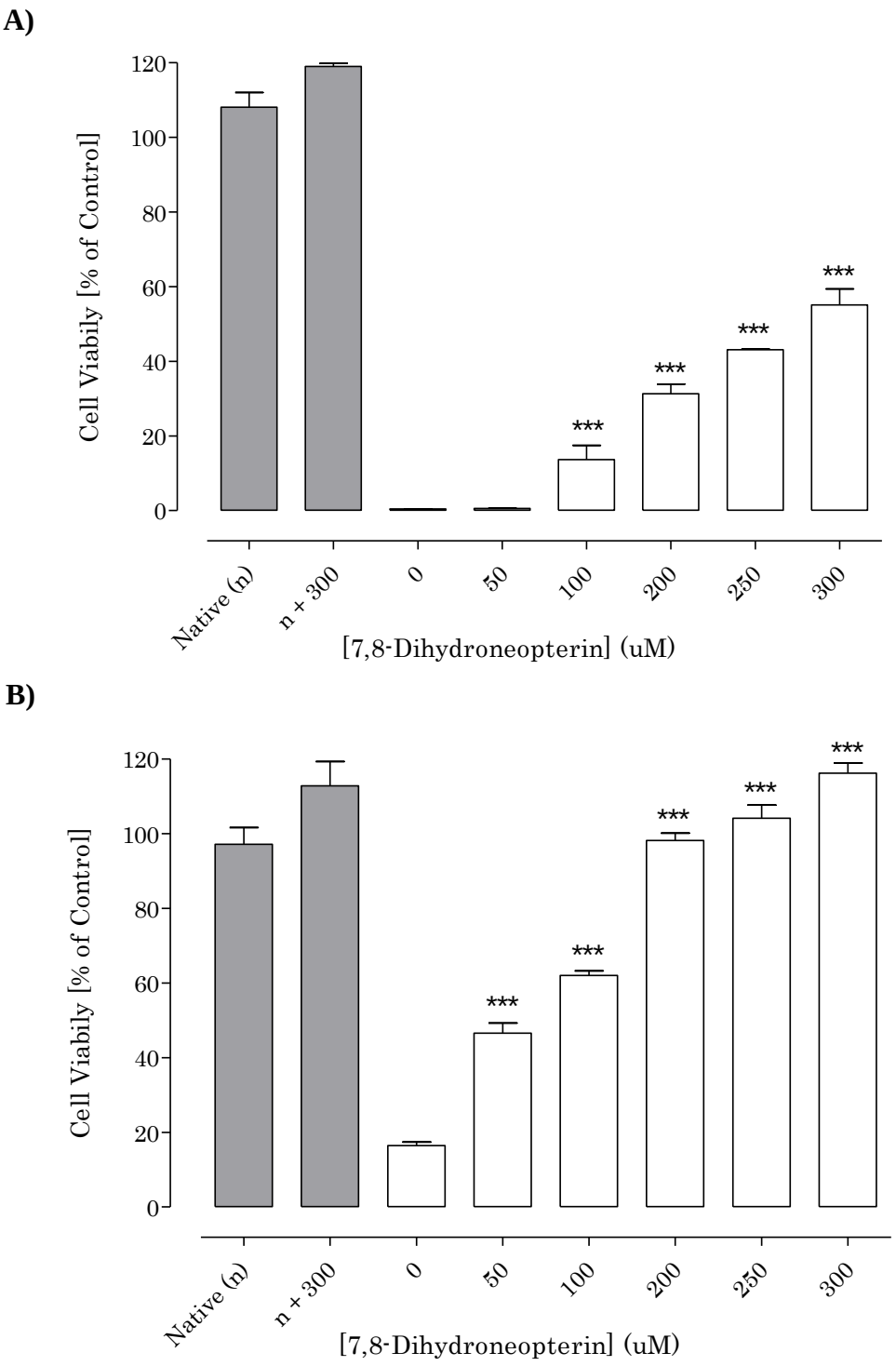


Figure 3.30 7,8-dihydroneopterin (7,8-NP) added to the incubation medium prevented cell viability loss in U937 cells upon exposure to oxALB. Cells (0.5×10^6 cells/ml) were pre-incubated in the presence or absence of varying concentrations of 7,8-NP in the dark, at 37°C for 15 minutes. Cells were incubated with or without 1 mg/ml of 1000/1 oxALB, that had been treated with **(B)** or without **(A)** methionine, over 24 hours. After incubation cells were washed and analysed using the MTT reduction assay. Viability was measured as a percentage of control containing the same cell treatment but with PBS in place of native or oxALB. Significance is indicated from the respective 0 μM 78-NP control on both graphs.

Having shown that 78NP does protect cells from cell death caused by oxALB, the next step was to assess whether this was due to a reduction in the intracellular oxidative stress. The primary function of GSH lies in reducing oxidative stress and maintaining the thiol-redox status of the cell (Forman *et al.*, 2009). GSH deficiency or a decrease in the GSH/glutathione disulfide ratio appears to result in increased susceptibility to oxidative stress or low cellular antioxidant capacity (Ballatori *et al.*, 2009), and is therefore a good measure of the presence of oxidative stress.

Incubation of U937 cells with oxALB caused a marked loss of intracellular glutathione (GSH) (figure 3.31b) closely reflecting the pattern of loss that was demonstrated for cell viability (figure 3.31a). In the absence of 78NP, GSH loss of more than 50% occurred following incubation with 1.0 mg/ml oxALB and almost complete GSH loss occurred at a concentration of 2.0 mg/ml oxALB. This experiment was performed in both the presence and absence of 78NP to examine its effect on GSH loss. As with cell viability, the presence of 78NP appeared to dramatically prevent oxALB-induced damage by way of GSH loss, and appeared to have the greatest effect at the lower end of oxALB concentrations used. At 0.5 mg/ml oxALB, the presence of 78NP caused slightly increased cellular GSH content compared to the native control, preventing GSH loss that occurred in its absence, at these concentrations of oxALB.

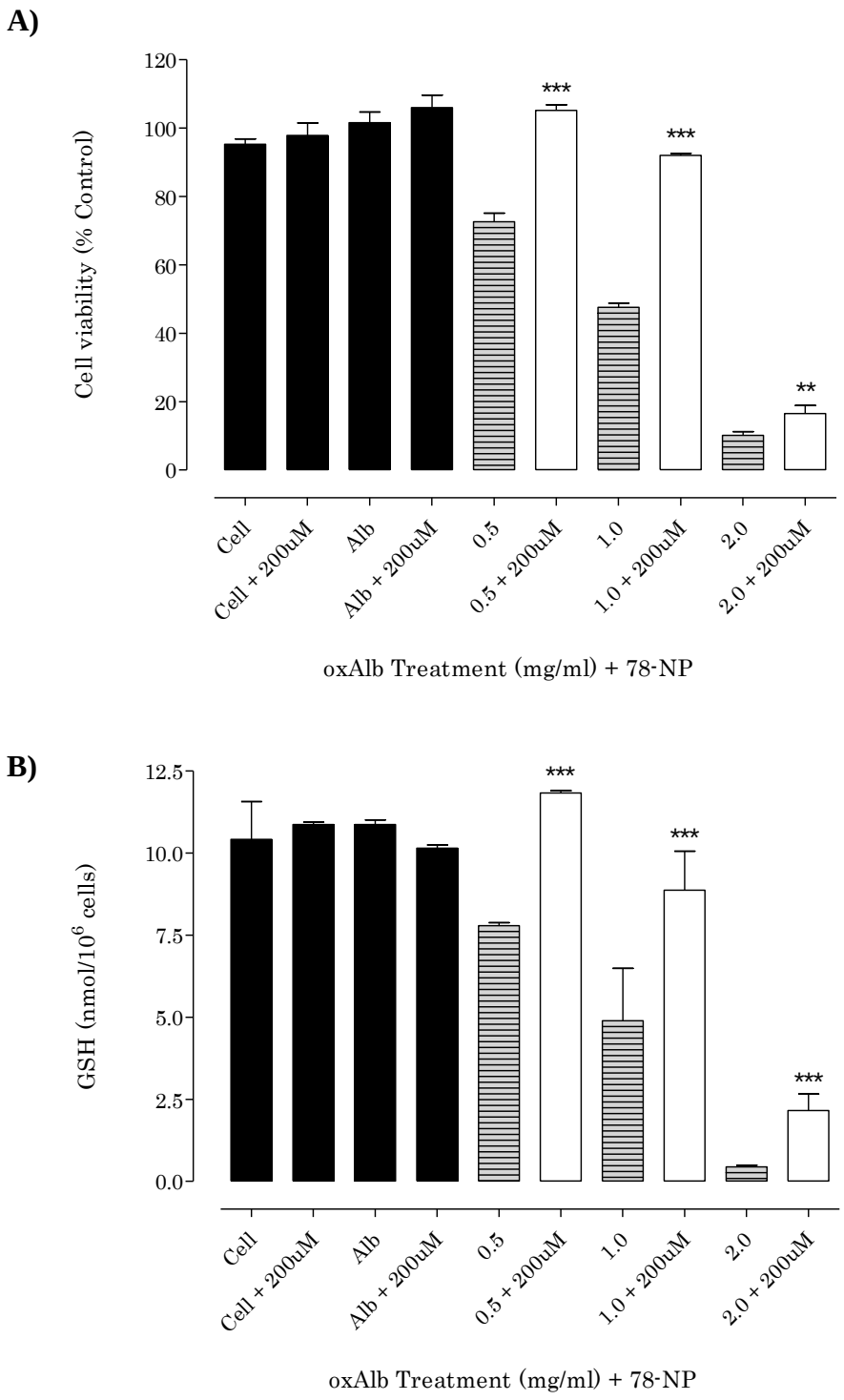


Figure 3.31 7,8-dihydroneopterin (7,8-NP) added to the incubation medium prevented GSH and cell viability losses in U937 cells upon exposure to oxALB. Cells (0.5×10^6 cells/ml) were pre-incubated in the presence or absence of 7,8-NP (200 µM) in the dark at 37°C for 15 minutes. Cells were treated with or without 1000/1 met-oxALB at 0.5 or 1.0 mg/ml over 24 hours. Cells were washed and analysed for viability using the trypan blue exclusion **(A),** or intracellular GSH **(B)** via HPLC analysis. Significance is indicated from the respective 0 µM 78-NP controls.

3.4.7 Kinetics of viability and glutathione loss upon insult with oxALB

Incubation of U937 cells with oxALB caused a marked loss of intracellular glutathione (GSH) (figure 3.33) closely reflecting the pattern of loss that was demonstrated for cell viability (figure 3.32). These experiments were performed in both the presence and absence of 78NP to examine its effect on the rate of viability and GSH loss.

The presence of 200 µM 78NP appeared to dramatically prevent almost all oxALB-induced viability loss, and appeared to have the greatest effect at 9 hours. 78NP caused a 65% increase in cellular GSH content at this time point compared to oxALB alone, preventing around 60% viability loss. In the absence of 78NP cell death occurred slowly over the first 6 hours before rapidly plummeting to 40% of the control viability between 6 and 9 hours (figure3.32).

The pattern of oxALB-induced GSH loss was studied over 24 hours using 1.0 mg/ml oxALB. This lethal concentration of oxALB caused a rapid loss of intracellular GSH content to 35% of cellular control within 6 hours of incubation and decreased to less than 18% of the initial intracellular GSH level during the next 18 hours (figure 3.33). Interestingly, the rapid loss in GSH, between 3 and 6 hours, preceded the dramatic loss of cell viability between 6 and 9 hours.

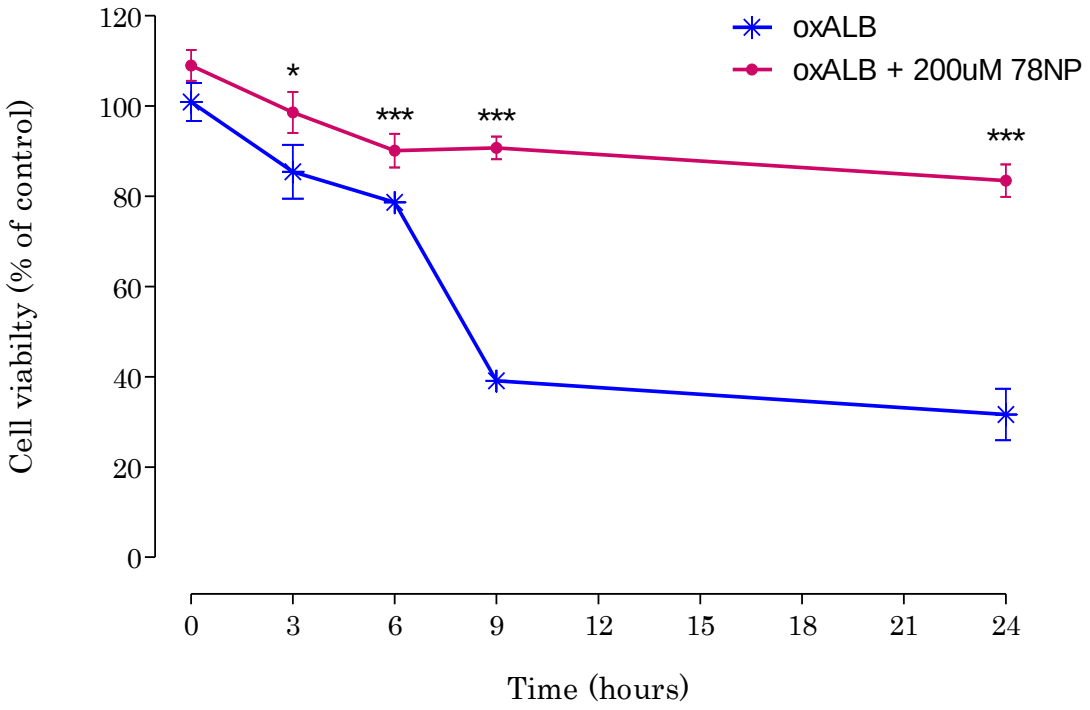


Figure 3.32 7,8-dihydroneopterin (7,8-NP) added to the incubation medium prevented cell viability loss in U937 cells upon exposure to oxALB. Cells (0.5×10^6 cells/ml) were pre-incubated in the presence or absence of 7,8-NP (200 µM) in the dark at 37°C for 15 minutes. Then cells were treated with or without 1 mg/ml 1000/1 met-oxALB over 24 hours. At set time points samples were taken out, cells were washed and analysed for viability using the MTT viability assay. Significance is indicated between treatments at each respective time point.

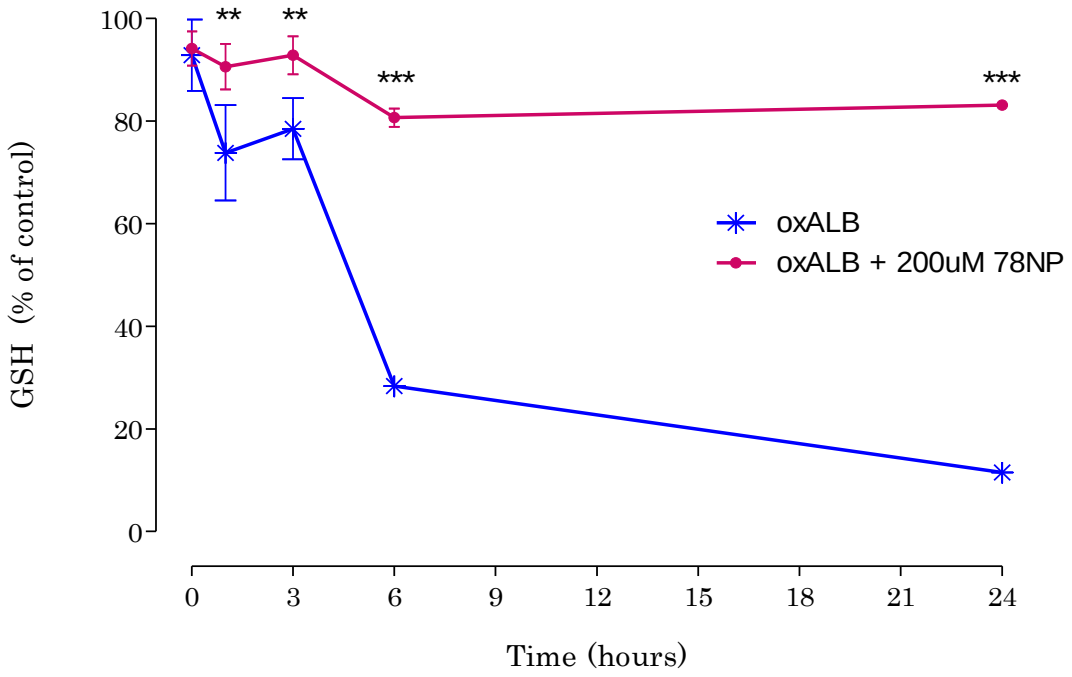


Figure 3.33 7,8-dihydroneopterin (7,8-NP) added to the incubation medium prevented intracellular GSH loss in U937 cells upon exposure to oxALB. Cells (0.5×10^6 cells/ml) were pre-incubated in the presence or absence of 7,8-NP (200 µM) in the dark at 37°C for 15 minutes. Then cells were treated with or without 1 mg/ml 1000/1 met-oxALB over 24 hours. At set time points, samples were taken out and analysed for intracellular GSH via HPLC analysis, [cell control value = 10.28 ± 1.13 Moles GSH/ Moles LDL]. Significance is indicated between treatments at each respective time point.

3.4.8 7,8-dihydroneopterin prevented oxidative stress upon exposure to oxALB

Previous data (intracellular GSH loss, figure 3.29; and protection by antioxidant 78NP, figure 3.30) suggests that oxALB-induced cell death is caused in part by an excessive rise in intracellular oxidative stress. DHE was utilised as a probe to examine superoxide production. It specifically reacts with superoxide anions to form a red fluorescent product to give a direct measure of oxidative stress within the cell. Cells were incubated with oxALB in the presence or absence of 78NP. During incubation cells were washed, probed with DHE and oxidative stress was quantified using flow cytometry. Each sample had 10,000 events recorded within a gate set to exclude cellular debris.

Comparatively, mean fluorescence intensity rises rapidly and peaks at 3 hours as with HOCl-oxLDL (figure 3.17). This can be observed (figure 3.34a) with the increasing size of the second peak to the right along the x-axis. As with cell viability and GSH loss, the presence of 78NP appeared to significantly reduce oxALB-induced damage by preventing oxidative stress (figure 3.34b). It appeared to have the greatest effect at 3 hours, preventing the largest rise in mean fluorescence seen with oxALB alone. It caused near complete retention of normal cell fluorescence as compared to a cell only control across the entire 24 hour incubation.

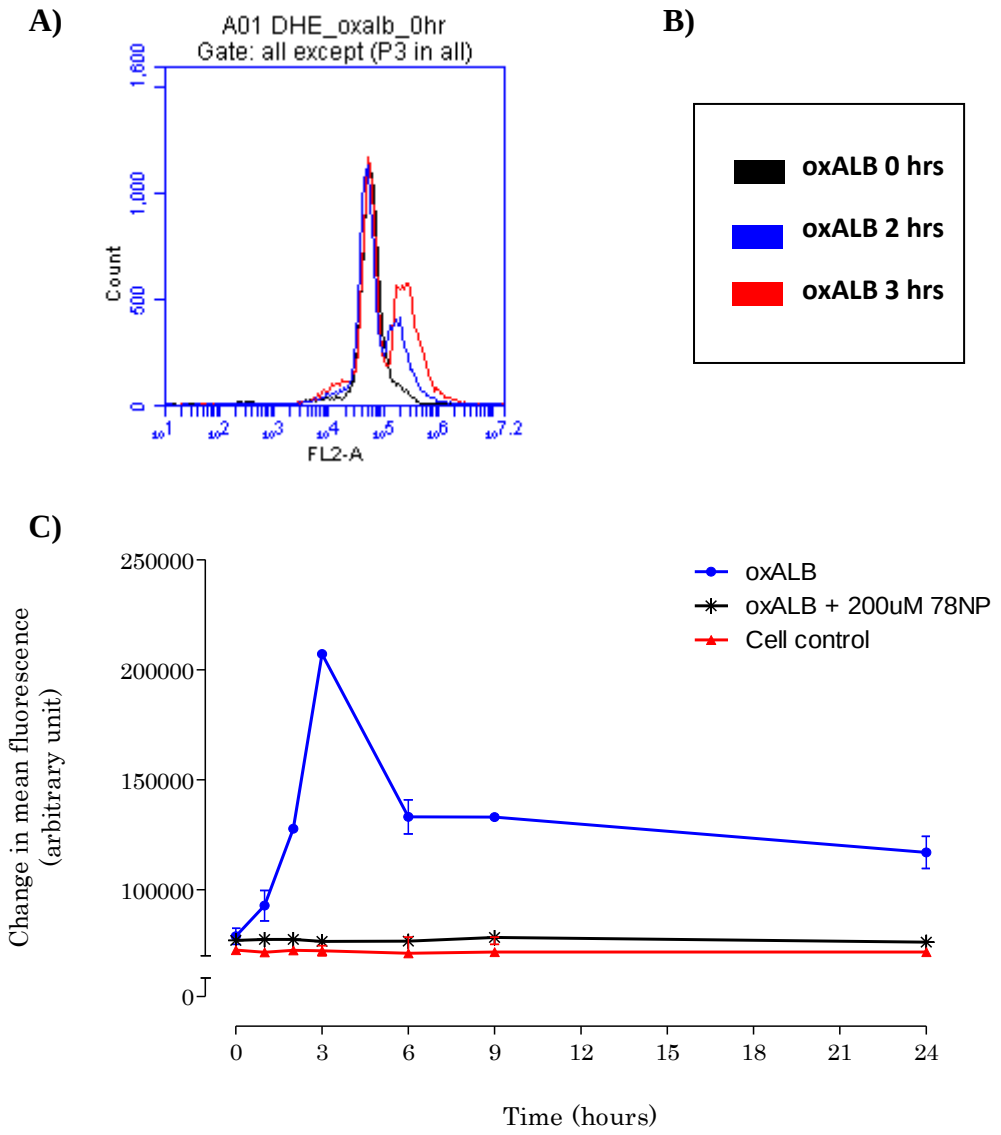


Figure 3.34 7,8-dihydroneopterin (7,8-NP) added to the incubation medium prevented oxidative stress in U937 cells upon exposure to oxALB. Cells (0.5×10^6 cells/ml) were pre-incubated in the presence or absence of 7,8-NP (200 µM) in the dark at 37°C for 15 minutes, then treated with or without 1 mg/ml 1000/1 met-oxALB over 24 hours. At set time points, samples were taken out and analysed for oxidative stress using DHE probe and flow cytometry through the FL-2 filter. Controls consisted of (0.5×10^6 cells/ml), RPMI and PBS in place of oxALB. The change in mean fluorescence over the first 3 hours can be seen through; an overlaid histogram **(A),** which can be deciphered using the legend **(B).** Alternatively all data points are shown in **(C).** Significance is indicated between treatments at each respective time point.

3.4.9 Detection of both apoptosis and necrosis in U937 cells following exposure to oxALB

The previous data clearly shows oxALB triggers cell death through oxidative stress and subsequent GSH loss. The nature of the cell death, whether it occurred by apoptosis or necrosis was investigated by flow cytometry. Cells (0.5×10^6 cells/ml) treated with 1.0 mg/ml 1000/1 oxALB over 24 hours. At set time points samples were taken out and analysed for cell death using AnnexinV-FITC (apoptosis) and PI (necrosis) probes using flow cytometry through FL3 and FL1 filters. Each sample had 10,000 events recorded within a gate set to exclude cellular debris. Mean fluorescence in the FL3 filter, measuring PI, only increased at 3 hours and each following measurement (figure 3.35a). Comparatively mean fluorescence in the FL1 filter, measuring AnnexinV-FITC, increased from 1 hour until peaking at 3 hours, before decreasing to control levels in subsequent time points (figure 3.35b). This suggests that there were separate populations of cells undergoing both apoptosis and necrosis at early time points before apoptosis is stalled, and cytotoxicity is due to secondary necrosis from around 6 to 24 hours.

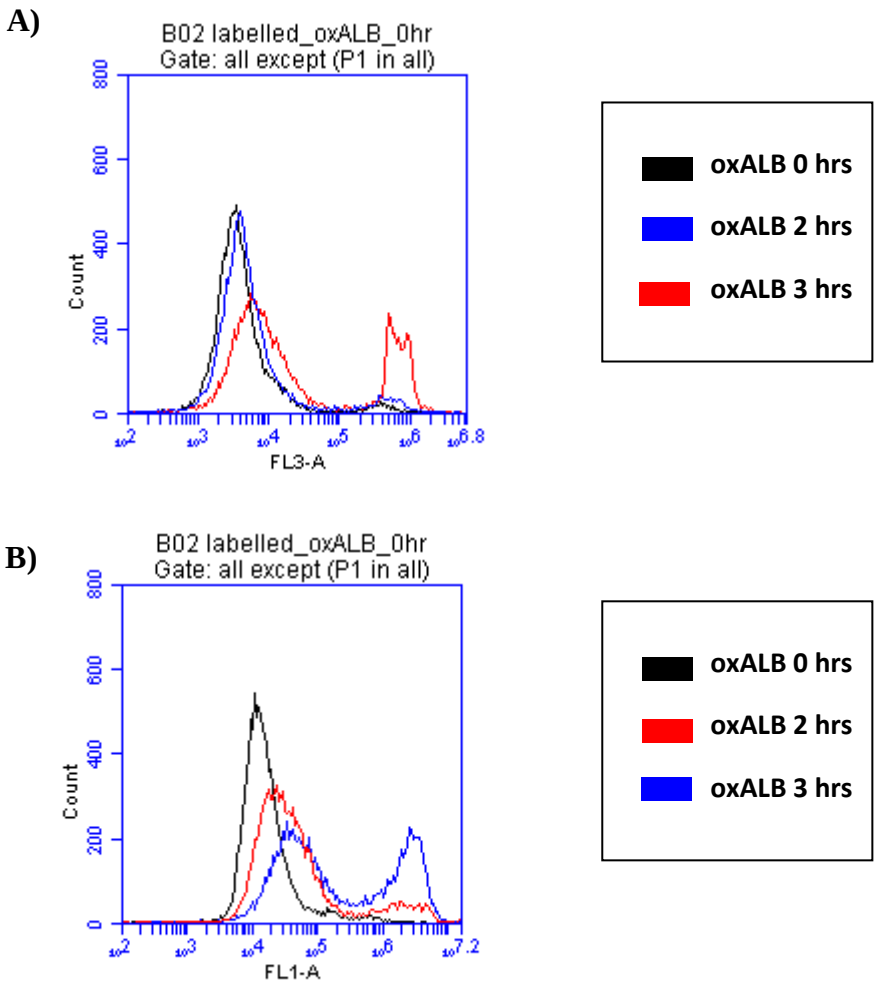


Figure 3.35 Apoptosis and necrosis follow after exposure of U937 cells to oxALB. Cells (0.5×10^6 cells/ml) treated with or without 1 mg/ml 1000/1 met-oxALB over 24 hours. At set time points samples were taken out and analysed for cell death using AnnexinV-FITC and PI probes using flow cytometry through FL3 and FL1 filters. The change in mean fluorescence for the necrosis probe (PI) over the first 3 hours can be seen through an overlaid histogram (**A)**. The change in mean fluorescence for the apoptosis probe (AnnexinV-FITC) over the first 3 hours can be seen through an overlaid histogram **(B)**.

4. DISCUSSION

4.1 Hypochlorous acid as a relevant oxidant in atherosclerosis

It is now well accepted that high plasma low-density lipoprotein (LDL) levels are a key risk factor in cardiovascular disease, and that oxidised LDL contributes to the pathology of atherosclerosis. There has been much interest in determining the precise nature of this oxidation, and identifying the agents responsible for it. Recently attention has focused on the role of phagocytic cells as a major source of reactive oxygen species, including the neutrophil, which produces large amounts of hypochlorous acid (HOCl) in a reaction catalysed by the highly abundant, heme enzyme myeloperoxidase (MPO) (Arnhold, 2004). There is a wealth of knowledge on myeloperoxidase and its product, HOCl and the evidence for their involvement in atherosclerosis is compelling. Catalytically active myeloperoxidase has been found in atherosclerotic lesions (Daugherty *et.al.*, 1994). Chlorinated tyrosine derivatives, which are thought to be specific products of MPO, have been detected during all stages of development of atherosclerosis (Heinecke, 1997), with elevated levels of 3-chlorotyrosine found in patients presenting with atherosclerotic complications (Hazen & Heinecke, 1997). Immunohistochemical studies have shown HOCl-generated protein oxidation products co-localized with macrophages and LDL in various lesion types (Hazell *et.al.*, 1996; Malle *et.al.*, 2000). However, many details regarding the mechanism, site of damage, and molecular events in the pathology remain to be answered.

A clear understanding of the products of HOCl-induced oxidation, together with analysis of oxidation products *in vivo* will allow evaluation of the role in atherosclerosis of myeloperoxidase, and the phagocytic cells that produce it. LDL contains both a protein and a lipid component, either of which may be involved in the oxidative damage during atherosclerosis, and consequently this study started by examining the effects HOCl had on this macromolecule.

4.2 Modification of LDL by HOCl

One important question arising from this study is whether HOCl concentrations utilised within this research were such that they favour HOCl-modification of lipoproteins *in vivo*, thus allowing appropriate pathophysiological conclusions to be drawn on the results. At sites of acute inflammation, HOCl concentrations are reported to be in the range of 340 μM or above (Katrantzis *et.al.*, 1991) which would result in a minimum molar oxidant/protein ratio of 250/1. Thus LDL was modified by incubating with varying oxidant/protein ratios ranging from 250/1 to 2000/1.

This laboratory has shown previously that protein hydroperoxide formation is tightly coupled to lipid oxidation during both copper- and AAPH-mediated LDL oxidation. Also, that THP-1 monocyte cell-mediated LDL oxidation induces the generation of significant levels of protein hydroperoxides (Gieseg *et al.*, 2003). Considering that protein hydroperoxides are found in atherosclerotic plaque (Fu *et al.*, 1998), are known to consume protective cellular thiols and ascorbate (Gebicki, 1997) and give rise to further radicals that damage other target molecules (Fu *et al.*, 1998, Gebicki, 1997), then evidence for a role of such protein oxidation products in oxLDL toxicity seems likely. Quantification of protein hydroperoxides (PrOOHs) formed from the oxidation of LDL with HOCl was carried out using the acetic acid FOX assay. PrOOH formation whilst increasing in a concentration dependent manner across varying HOCl/LDL molar ratios (figure 3.4a), did not exhibit anywhere near the yield found from the oxidation of LDL with AAPH (Gieseg *et.al*, 2003). This may be due to the fact that reactions with HOCl are very rapid and measurements of PrOOH were conducted after the hydroperoxides had already stated to decay, although this is not likely as Simpson *et.al.*, (1992) found their half-lives to be 5 days, and a subsequent experiment showed that the levels of PrOOH actually increased with time (figure 3.4b). A more probable scenario is that the radicals generated from HOCl oxidation with LDL failed to produce peroxyl radical (indicating no interaction with poly unsaturated fatty acids), hence no carbon centred radical was formed, which is integral in the series of reactions that occurs in PrOOH formation.

To determine whether the protein moiety of LDL was being oxidised by HOCl an alternative measure of protein oxidation was utilised. Tyrosine loss is an indicator of general protein damage. HOCl caused tyrosine loss in a concentration-dependent manner (figure 3.1). Tyrosine is relatively low on the reactivity series of HOCl, therefore if tyrosine is oxidised one can assume that other amino acids are also being oxidised, and thus the protein moiety effected in the oxidation of LDL with HOCl.

With the majority (80%) of the LDL molecule made up of cholesterol and lipids, the protein moiety is by far not the only available target for HOCl. A common marker of lipid peroxidation is the formation of thiobarbaturic acid reactive species (TBARS), and high levels have been found in Cu-oxidised LDL. Although TBARS did increase with HOCl oxidation, comparatively the levels were negligible to those produced with Cu-oxLDL (figure 3.5). It has been shown that HOCl has the ability to break down malondialdehyde (MDA) (Winterbourn *et.al*., 1993) a key component to the TBARS assay and may explain why there were such low levels of TBARS produced. If this is the case then this does not necessarily suggest that lipid peroxidation is not occurring, simply the validity of the assay is questionable under these parameters, and an alternative method of lipid peroxidation should be assessed.

An exploration of the lipid composition of Cu-oxLDL and of a macrophage foam-cell model generated by the uptake of oxLDL demonstrated that when LDL undergoes oxidation *in vitro* a number of changes in lipid composition occur, including the substantial loss of free and esterified cholesterol and the generation of oxysterols (Brown *et al.*, 1996). Oxysterols are the 27-carbon products of cholesterol oxidation and have been widely implicated in the development of atherosclerosis. During the copper-mediated oxidation of LDL *in vitro*, it has been shown that up to 50% of the cholesterol is converted to oxysterols, with the major oxysterol present as 7-ketocholesterol (Brown *et al.*, 1996). Unlike the previous studies with Cu-oxLDL, only trace amounts of 7-ketocholesterol (7KC) were detected when LDL was oxidised with HOCl (figure 3.8). This result, along with the lack of interaction with polyunsaturated fatty acids, demonstrates clearly that the lipid component of the LDL molecule is not the main target of attack for HOCl.

Given that lipid peroxidation products have been found in atherosclerotic lesions (Brown & Jessup *et.al.*, 1999) and elevated levels of 7-ketocholesterol have been linked to cell death within the plaque (Lizard *et.al.*,1999), it was of interest to determine if HOCl-oxLDL underwent secondary reactions with free metal ions present in the incubation medium, RPMI-1640; especially after the removal of HOCl and the complete loss of the antioxidant α-tocopherol (figures 3.2 & 3.9). Surprisingly, the minute levels of TBARS went down after the 24 hour incubation in RPMI-1640 medium. Whilst this is probably due to the prolonged exposure to HOCl and breakdown of MDA, no definite conclusions could be made due to the limitations of this assay. Were TBARS actually decreasing overtime due to its half-life and/or forming further by-products, or were TBARS not detected because the MDA had reacted with HOCl giving a false negative result? 7-ketocholesterol levels did increase; however, levels were still significantly lower than those found with copper-mediated LDL oxidation. It would be interesting to see whether 7KC levels did continue to increase over time during a longer incubation period, and whether removal of metal ions still allowed for further lipid peroxidation. It would also be of interest to use a more oxidative permissive media such as Ham's F-10 in further studies. This would substantiate the hypothesis for a role protein oxidation products play in the propagation of lipid peroxidation

OxLDL plays a crucial role in atherosclerosis as a cause of foam cell formation, cell proliferation and migration, adhesion of endothelial cells and extracellular matrix propagation at atherosclerotic lesions. It has long since been established that it is also involved in the advancement of atherogenesis as a cause of cell death within the necrotic core. Cu-oxLDL has been shown to be toxic to several cell types including: smooth muscle cells (Ding *et.al.*, 2011), endothelial cells (Kuzuya *et.al.*, 1990), U937s (Baird *et.al.*, 2004), THP-1s (Namgaladze *et.al.*,2008) and macrophages (Baird *et.al.*, 2005). The cause of this toxicity is subject to vigorous debate. Many propose that the toxic agent within the oxLDL is the lipid-peroxidation product 7-ketocholesterol. However, a recent study from this laboratory has found that to be unlikely (Rutherford & Gieseg, 2012), and brought the involvement of 7-Ketocholesterol in the toxicity of oxLDL into question. This raises the question of whether the toxic agent is a protein oxidation product.

4.3 Toxicity of HOCl-oxLDL

The U937 human monocytic cell line was selected for use in this model system since monocytes are involved in the primary and on-going interactions with modified lipoproteins within the artery wall (Lusis, 2000). Importantly, this laboratory has previously shown that the Cu-oxLDL-induced cell death mechanism in U937 cells is the same as that seen in human monocyte-derived macrophages prepared from human blood (Baird *et al.*, 2004). In both cell types, Cu-oxLDL causes large reactive oxygen species-induced oxidative stress, loss of cellular glutathione and oxidative loss of regulatory metabolic enzymes, triggering caspase-independent necrosis (Gieseg *et al.*, 2009b). U937 cells are a commonly used atherosclerotic model since they allow for the investigation of relatively homogeneous groups of cells, compared to the more heterogeneous nature of comparatively small quantities of human monocyte-derived macrophages isolated from human blood samples. U937 cells have a doubling time of between 3-4 days depending on the presence of serum in the cell culture medium. The use of a rapidly growing cell line over relatively long incubation periods means that cell growth must be taken into consideration during experimental design, for instance conducting toxicity experiments on cells in the same cell cycle. For this reason, experiments were designed such that the effects of cell proliferation were minimised, for example, data were expressed as a percentage of, or relative to cell-only control treatments.

This investigation began by examining the effect of HOCl-oxLDL on U937 cells, in terms of cell viability and glutathione content. HOCl-oxLDL showed a high degree of toxicity to U937 cells, with the median lethal dose found to be between 0.5-1.0 and up to 1.25 mg/ml oxLDL, depending upon the individual oxLDL preparation. The concentrations of HOCl-oxLDL used in the experiments are within the range of LDL concentrations *in vivo*, as serum concentrations of LDL in normolipidemic persons are maintained at approximately 3.0 mg/ml (Esterbauer *et al.*, 1992).

There was no significant difference in cell viability loss between varying oxidant/protein molar ratios, rather there was increased loss with increased concentrations of HOCl-oxLDL given to cells (figure 3.11). This suggests that the cause

of the toxicity from HOCl-oxLDL is bound to the molecule and has become saturated by the HOCl even at the lowest HOCl/LDL molar ratio. It is the presence of more of the HOCl-oxLDL molecules (carrying the toxic component) that is the cause for increased toxicity.

Time course studies indicated that the loss of cell viability caused by HOCl-oxLDL occurred rapidly, between 6 and 9 hours (figure 3.16a). This progression of viability loss was very closely correlated with loss of cellular glutathione content (figure 3.16b), although glutathione loss appeared to occur slightly ahead, and more rapidly than viability loss. Glutathione loss provides a direct measure of the cellular antioxidant status and it has been established previously that cellular glutathione loss results in a further increased susceptibility to oxidative stress (Ballatori *et al.*, 2009). HOCl-oxLDL clearly induces an oxidative stress in U937 cells, causing reduced glutathione content in parallel with reduced cell viability, which is consistent with previous findings by this laboratory (Baird *et.al.*, 2004).

In the case that 7-ketocholesterol was the toxic agent within oxLDL, one would expect HOCl-oxLDL with low-levels of 7-ketocholesterol not to be cytotoxic. This study found otherwise, which suggests that we need to look elsewhere to determine the cytotoxic mechanism of HOCl-oxLDL.

4.4 Mechanism of oxLDL toxicity

In exploring the mechanism of action of HOCl-oxLDL to U937 cells, it is necessary to consider firstly whether HOCl-oxLDL is actually internalised by cells. Although there has been debate as to whether U937 cells express scavenger receptors necessary for the unregulated uptake of HOCl-oxLDL, it is now widely accepted that they do. The CD36 receptor (belonging to scavenger receptor class B) has been shown to internalise HOCl-oxLDL in U937 cells (Pietsch *et al.*, 1996). Expression of class A scavenger receptors has been shown to occur during PMA-induced differentiation of U937 monocytes to macrophages (Banka *et al.*, 1991, Shimaoka *et al.*, 2000) and differentiation of U937 cells by 0.1 mg/ml oxLDL was shown to induce scavenger receptor expression (Lei *et*

al., 2002) which indicates that HOCl-oxLDL is actively absorbed by U937 cells. The current hypothesis for Cu-oxLDL cytotoxicity is that it causes high levels of intracellular oxidative stress, including the formation of highly reactive oxidised lipid and protein species that overwhelm the cells' antioxidant capacity, inducing imbalances in the intracellular redox environment, changes in various signalling pathways and gene expression, leading to cell death via caspase-independent necrosis (Baird *et.al*, 2004).

Chloramines are a significant by-product of HOCl interaction with proteins, and are known to be toxic towards many cell types (Tatsumi & Fliss, 1994; Thomas, 1979). Given that the previous results in this study show that the protein component of LDL is the main target of HOCl attack, removal of chloramines after oxidation of LDL, surprisingly did not affect the toxicity of HOCl-oxLDL (figure 3.13). This suggests that chloramines were not present in the HOCl-oxLDL solution and not the cause of HOCl-oxLDL toxicity. This could be due to a number of reasons: either no chloramines were formed because there was little to no accessibility of amine targets on the LDL molecule during HOCl attack; or chloramines have broken down either back to amines or onto reactive aldehydes. The latter is more plausible as previous studies have found chloramine formation upon HOCl-oxLDL (Hazell & Stocker, 1993). Chloramines although relatively stable at 4 ˚C, are rapidly lost with a half-life of 100 minutes in PBS at 37 ˚C (Hazell & Stocker, 1993). Since the oxidation method and subsequent dialysis of LDL occurs for 24 hours before chloramines removal, it is more than likely there were no chloramines to remove, but broken down to reactive aldehydes, which themselves are cytotoxic (Hazen *et.al.*, 1996), and could contribute to the toxicity of the HOCl-oxLDL.

For cells to function properly at inflammation sites, including in atherosclerotic lesions, they need to possess antioxidative mechanisms defending themselves against oxLDL induced stress. The physiological role of glutathione is multi-faceted and has been implicated in a multitude of cellular functions, such as the transport of amino acids, synthesis of proteins and nucleic acids, and maintenance of enzymes in active forms (Pastore *et al.*, 2003). Glutathione (GSH) is the major intracellular antioxidant and its deficiency has been implicated in a number of diseases, possibly due to the increased susceptibility of cells/tissues to oxidative stress (Ballatori *et al.*, 2009). The antioxidant

function of GSH depends primarily on its role as a component of the enzymatic pathway that cells developed against ROS. GSH provides a first line of defence against ROS, as it can scavenge free radicals and reduce H_2O_2 (Pastore *et al.*, 2003). Moreover, GSH is significantly lost in red cells (Vissers & Winterbourn, 1995) and neutrophils (Carr & Winterbourn, 1997) before cell lysis occurs, indicative of its role in defending cells against oxLDL.

It was found that U937 cells treated with HOCl-oxLDL showed significant cell viability loss (between 6 and 9 hours) after the intracellular GSH level was significantly decreased (between 3 and 6 hours) (figures 3.14 & 3.16). This implies that HOCl-oxLDL did not react with cell membranes first causing membrane integrity loss, but penetrated the plasma membrane to react with intracellular GSH first. Morphological examination of the cells at each time point showed that the cells began to swell and underwent necrotic cell death rather than apoptosis. The above results also imply that once GSH was no longer abundant enough in cells to effectively scavenge HOCl-oxLDL, the remaining HOCl-oxLDL then reacted with other biomolecules, causing membrane integrity loss and cell death. This hypothesis is consistent with previous studies, which showed significant Cu-oxLDL-induced intracellular GSH loss before cell lysis or cell swelling were observed (Baird *et.al.*, 2004).

Oxidative modification of lipids alters their physical properties to such an extent that they may undermine the structural integrity of the membrane. Most cells have an impressive arsenal of antioxidant compounds and enzymes at their disposal that protects against the generation and accumulation of lipid oxidation products in their membranes. Along with GSH mentioned above, 7,8-dihydroneopeterin is a relevant antioxidant to study for its role it plays in inflammation and potentially atherosclerotic lesions. Human macrophages can synthesize and release neopterin and its reduced form, 7,8-dihydroneopterin (78NP), upon induction by interferon-γ (IFN-γ) (Wachter *et al*., 1989; Wachter *et al*., 1992). 78NP may be produced and play a role within the lesions, and has been detected in atherosclerotic lesions of humans (Hansson *et al*., 1989; Libby, 1995; Zhou *et al*., 1998). In addition, neopterin levels are found to be significantly elevated in patients with vascular disease (Schumacher *et al*., 1992; Tatzber *et al*., 1991; Rudzite *et al*., 2003). It has been hypothesized that 78NP is synthesised by γ-IFN-stimulated

macrophages to protect the cells against the oxidants encountered within an inflammatory site; including oxLDL (Gieseg *et al*. 2007). U937 cells were fully protected from 1000/1 HOCl-oxLDL induced cell viability and glutathione loss by addition of 250 μM 78NP (figures 3.15 & 3.16). This indicates that 78NP scavenged HOCl-oxLDL efficiently to prevent it from oxidizing intracellular GSH and causing cell death. This is in agreement with previous studies, which showed that 78NP is a potent antioxidant scavenger and protects U937 cells and HMDM cells from Cu-oxLDL cellular damage (Baird *et al.*, 2004, Baird *et al.*, 2005). The effective concentrations of 78NP added to cells in the above experiments appeared to be higher than the *in vivo* neopterin concentrations reported. Up to 2 μM neopterin has been reported in atherosclerotic plaques from carotid artery (Gieseg *et al.*, 2007). However, the 78NP concentrations in the local environment of cells are likely to be higher than the above reported value.

With the previous findings that suggest a role for reactive oxygen species in Cu-oxLDL cell death, DHE was measured to determine whether reactive oxygen species represent a cause or product of HOCl-oxLDL-induced cell death. DHE fluorescence rose steadily from initial contact with HOCl-oxLDL and peaked at 3 hours. This is preceded GSH loss and subsequent viability loss. 78NP did protect U937 cells from the oxidative stress induced by HOCl-oxLDL (figure 3.17) which suggests that intracellular oxidative stress is a key factor in driving cell death.

4.5 Protein oxidation in atherosclerosis

In a review of oxidative modifications (Stocker & Keaney, 2004), it was highlighted that the correlation between levels of oxidised lipids and lesion development, as predicted by the widely accepted oxidative modification hypothesis, is not observed in either *ex vivo* or *in vitro* studies. Together with the results of this study, it seems appropriate that alternative mechanisms of oxLDL cytotoxicity be considered. Although there is solid evidence to suggest that lipid oxidation and toxicity are linked (Clare *et al.*, 1995), indications that protein oxidation may play an important role in cytotoxicity are numerous (Hazell *et al.*, 1996, Stocker & Keaney, 2004, Upston *et al.*, 2002). This

provides an opening for the suggestion that the products of protein oxidation may be at the heart of oxLDL-induced cell death and the development of the necrotic core.

Of direct relevance is an investigation that aimed to distinguish and examine processes resulting from the direct oxidation of protein (as compared to the derivatisation of lysine residues on apoB-100). The study examined which oxidative species might contribute to protein-bound oxidation products such as chlorotyrosine, dityrosine, 3,4-dihydroxyphenylalanine (DOPA), o-tyrosine and m-tyrosine, which are detected in advanced atherosclerotic plaques. Their findings indicated that hydroxyl radical-induced damage to protein is a plausible influence in atherosclerotic plaque (Fu *et al*., 1998). Their findings also raised the issue of the source of hugely increased dityrosine levels that are measured in atherosclerotic plaques. Levels of dityrosine (a well-established hallmark for oxidised protein levels) have been shown to increase significantly from the early to late stages of plaque development. It has also been established by Heinecke *et.al.* (1993) that tyrosine can act as a substrate for MPO-derived HOCl, resulting in the formation of a tyrosyl radical which is then able to cause oxidation of cholesterol or the cross-linking of proteins via dityrosine formation (Savenkova *et.al.*, 1994).

Further to this, hypochlorite (HOCl)-modified proteins that have been identified in human atherosclerotic plaque have been shown to transform LDL to a high-uptake form without significant lipid oxidation (Hazell *et al*., 1996). The accumulation of oxidised modifications may decrease the proteolytic susceptibility of some portions of oxidised protein (Grant *et al.*, 1993). This is particularly significant in the case of long-lived resident macrophage cells, where the accumulation of reactive protein oxidation products such as advanced oxidation protein products (AOPPs) may cause further damage to other cellular macromolecules in the vicinity.

4.6 Modification of Albumin by HOCl

Advanced oxidation protein products (AOPPs) are dityrosine-containing, cross-linked protein products formed during the oxidative burst of monocytes via myeloperoxidase reaction, and are carried mainly on albumin in the plasma (Witko-Sarsat *et.al.*, 1996;

Witko-Sarsat *et.al.*, 1998). AOPPs are not innocent end products of the activation of macrophages, they are biologically active molecules capable of mediating oxidative stress and respiratory burst in monocytes (Witko-Sarsat *et.al.*, 1996). AOPPs are capable of inducing/accelerating renal injury in the tubular cells, via the CD36 pathway (Iwao *et.al.*, 2006), and in vascular endothelial cells, via a receptor for advanced glycation end-products (RAGE)-mediated signalling pathway (Sebekova *et.al.*, 2012). With the aim of creating a physiologically relevant model for testing the toxicity of AOPPs, bovine serum albumin was incubated with HOCl at varying oxidant/protein molar ratios ranging from 250/1 – 2000/1. Increasing HOCl/BSA molar ratios bands on SDS-PAGE gels became increasingly blurred and fragmented (figure 3.18). There was also significant loss of tyrosine residues coinciding with an increase in oxidative tyrosine products, including dityrosine (figure 3.19). Although these results suggest that AOPPs have formed (and validate the oxidation of BSA as a relevant model in studying AOPPs), it cannot be determined for certain, so the oxidative product was termed oxALB.

4.7 Toxicity of oxALB

OxALB caused a concentration-dependent loss of cell viability (figure 3.22). This is in agreement with a number of studies using different cell types including U937 cells (Vicca *et al*., 2003; Ermak *et al*., 2010) and THP-1 cells (Vicca *et.al.*, 2000). In contrast to the cytotoxic effects of HOCl-oxLDL, oxALB displayed significant differences between increasing HOCl/BSA molar ratios, which was especially emphasised on oxALB that had undergone dialysis treatment (figure 3.21). The precise toxicity of oxALB towards U937 cells was found to vary between oxALB preparations, however this difference was minimized after dialysing residual HOCl that had not reacted from the oxALB solution.

Comparisons of toxicity between HOCl-oxLDL and oxALB were determined by incubating cells with equivalent total mass (1.0 mg/ml) or equivalent protein mass (1.0 mg/ml HOCl-oxLDL and 0.2 mg/ml oxALB). OxALB (1.0 mg/ml) displays a high level of toxicity to U937 cells, comparable to that of Cu-oxLDL, and HOCl-oxLDL

(figure 3.23). OxALB was significantly less toxic than HOCl-oxLDL when exposed to U937 cells at equivalent protein mass (figure 3.24). HOCl-oxLDL and oxALB more than likely share a similar toxic protein component. However, HOCl-oxLDL also possesses a significant amount of lipids, which although not initially a target of HOCl attack could undergo peroxidation propagated by oxidative protein products formed from the initial HOCl exposure, and may explain the difference in toxicity.

Cell viability was measured by both MTT reduction assay and trypan blue exclusion assay (figure 3.28). The MTT reduction assay measures metabolic activity of cells; MTT compound is reduced by mitochondrial NADH dehydrogenases to formazan only in living, metabolically active cells (Mosmann, 1983). Yet, stimulated cells can produce more formazan than resting cells, suggesting that the observed cell viability loss could be attributed to either an increased ratio of dead to viable cells or decreased metabolic activity in the viable cells. Cell viability measured by the trypan blue exclusion assay (monitors cell membrane integrity) agrees with that obtained by the MTT assay. This indicates the observed cell viability loss was indeed due to cell death. Irrespective of their different mechanisms for detecting cell death, these two assays provided strong corroboration of all viability results obtained.

The time course of oxALB-induced cell viability loss showed that all viability loss occurred rapidly during the first 6 hours (figure 3.28). The rapid metabolic energy loss (measured by MTT assay) and perturbation of cellular membranes (seen though swelling and lysis of cell in the trypan blue assay) implied that oxALB induced necrotic cell death in U937 cells.

4.8 Mechanism of oxALB toxicity

Removal of chloramines reduced the toxicity of oxALB (figure 3.26) significantly but not completely, this was especially apparent when cells were exposed to higher concentrations of oxALB. The reason for this seems to be some biphasic toxic mechanism. Chloramines appear to be responsible for this toxicity, but cannot be attributed for the entirety of the toxic mechanism of oxALB. This anomaly could be due

to the reactivity of chloramines as they retain sufficient oxidizing capacity to directly modify biomolecules. In addition, chloramines can break down to form reactive aldehydes or nitrogen-centered radicals (Carr *et.al.*, 2000). They themselves are cytotoxic and may be responsible for the remaining toxicity of oxALB after the removal of chloramines. Because the half-lives of chloramines are relatively short (see section 4.4) it would be interesting to see if the toxic agent of oxALB is due to the initial chloramine production and subsequent aldehyde formation. One way to examine this may be to add methionine directly to oxidation solution, but methionine is highly reactive with HOCl (Winterbourn, 1985; Pattison & Davies, 2001) and would act as a scavenger reducing the HOCl attack on the BSA. By oxidising BSA at 4˚C it will mitigate the reactivity of chloramines, which are stable and unreactive (3 day half-lives) at this lower temperature (Hazell & Stocker, 1993). Although this would not be physiologically relevant as it would not mimic the conditions of HOCl oxidation found *in vivo*. This may be a dilemma which cannot be resolved by the modification of the oxidation process but rather experimentation with further analytical techniques.

U937 cells treated with oxALB showed rapid GSH loss between 3 and 6 hours (figure 3.33). A point of similarity between the two mechanisms (HOCl-oxLDL and oxALB) of toxicity is that the viability loss was preceded by a rapid loss in GSH. Such a close relationship between cellular glutathione loss and viability loss generally indicates the involvement of radical oxygen species. DHE was utilised as a probe to determine whether oxidative stress increased upon exposure to oxALB. As with HOCl-oxLDL, oxALB induced an increase in DHE fluorescence which rose steadily, peaking at 3 hours (figure 3.34). It appears that oxALB induced cell death of the U937 cells in the same manner as HOCl-oxLDL, by inducing oxidative stress, leading to a rapid GSH loss and subsequent viability loss. In addition 200 µM 78NP could prevent all of these effects from occurring and completely prevented cell death (figures 3.30, 3.31, 3.32, 3.33 & 3.34).

Research directed at understanding the functional consequences of cell death in atherosclerosis has revealed opposing roles for cell death in atherosclerotic plaque progression. In early lesions, apoptosis limits lesion cellularity and suppresses plaque progression (Liu *et.al.*, 2005; van Vlijmen *et.al.*, 2001). In advanced lesions, cell death

promotes the development of the necrotic core, a key factor in rendering plaques vulnerable to disruption and acute lumenal thrombosis (Seimon & Tabas, 2009). Apoptosis seems to be clearly advantageous, because the elimination of the apoptotic cells or resulting apoptotic bodies by phagocytosis prevent the release of intracellular content and consequent damage of the surrounding tissue, as it occurs in necrosis. Therefore discovering the type of cell death that occurs when cells are exposed to oxALB is of great importance in determining the role oxALB may play in the advancement of atherogenesis.

Flow cytometric analysis of phosphatidylserine exposure, detected by AnnexinV-FITC binding, showed the cells were becoming apoptotic in the first 3 hours of incubation with oxALB (figure 3.34), coinciding with the peak in ROS. After 3 hours little to no apoptosis staining was detected. OxALB caused a rapid increase of propidium iodide (necrosis marker) in U937 cells after GSH depletion at 6 hours (figure 3.34). This result was in agreement with the earlier results where morphological changes were observed, which demonstrated that oxALB caused U937 cell swelling and cell membrane rupture. The suppression of apoptosis and the induction of necrosis was observed when depletion of GSH occurred. An increase in intracellular oxidation may determine the selection between apoptosis and necrosis. Nagai *et al*., (2002) have reported that GSH depletion causes necrosis in mouse hepatocytes. Apoptosis is an energy-dependent process and the decrease in ATP below critical levels prevents the execution of apoptosis whilst promotes necrosis. The results of this work suggest that changes in GSH status play a critical role in the mechanisms that regulate apoptosis and necrosis. OxALB clearly has the potential to play a major part to play in cell death. Protein oxidation products may be responsible for not only cell death in atherosclerotic lesions, but may also propagate lipid peroxidation resulting in further oxidative stress and cellular death, enhancing the necrotic core causing destabilization of the plaque.

4.9 Summary

It appears that HOCl reacts preferentially with the protein moiety of LDL, and it is hypothesised that lipid damage takes place more slowly via a secondary reaction involving the breakdown of protein-based products. The few studies carried out that investigate the kinetic progression of such damage show that protein oxidation is an early event in ROS-initiated events (Du & Gebicki, 2004). The derivatisation of protein is known to occur at the same time that LDL becomes toxic and also correlates with the conversion of LDL to the high-uptake form (Du & Gebicki, 2004).

HOCl-oxLDL generated in such a manner, with a near absence of oxysterols, displays a high level of toxicity to U937 cells, comparable to that of Cu-oxLDL. HOCl-oxLDL and oxALB caused concentration-dependent losses of cell viability. Rapid metabolic energy loss (measured by MTT reduction assay), cell membrane integrity loss, cell membrane rupture, and cell swelling were observed (measured by trypan blue exclusion assay). This implies that HOCl-oxLDL and oxALB caused U937 cells to undergo necrotic cell death. Depletion of intracellular GSH rendered cells more sensitive to necrosis. These results indicate that intracellular GSH played an important role in protecting U937 cells from HOCl-oxLDL and oxALB induced oxidative stress. Exposing U937 cells to HOCl-oxLDL and oxALB in the presence of 78NP significantly reduced intracellular GSH and cell viability loss, indicating that 78NP was an efficient scavenger of HOCl-oxLDL and oxALB and out-competed intracellular GSH for HOCl (see figure 4.1).

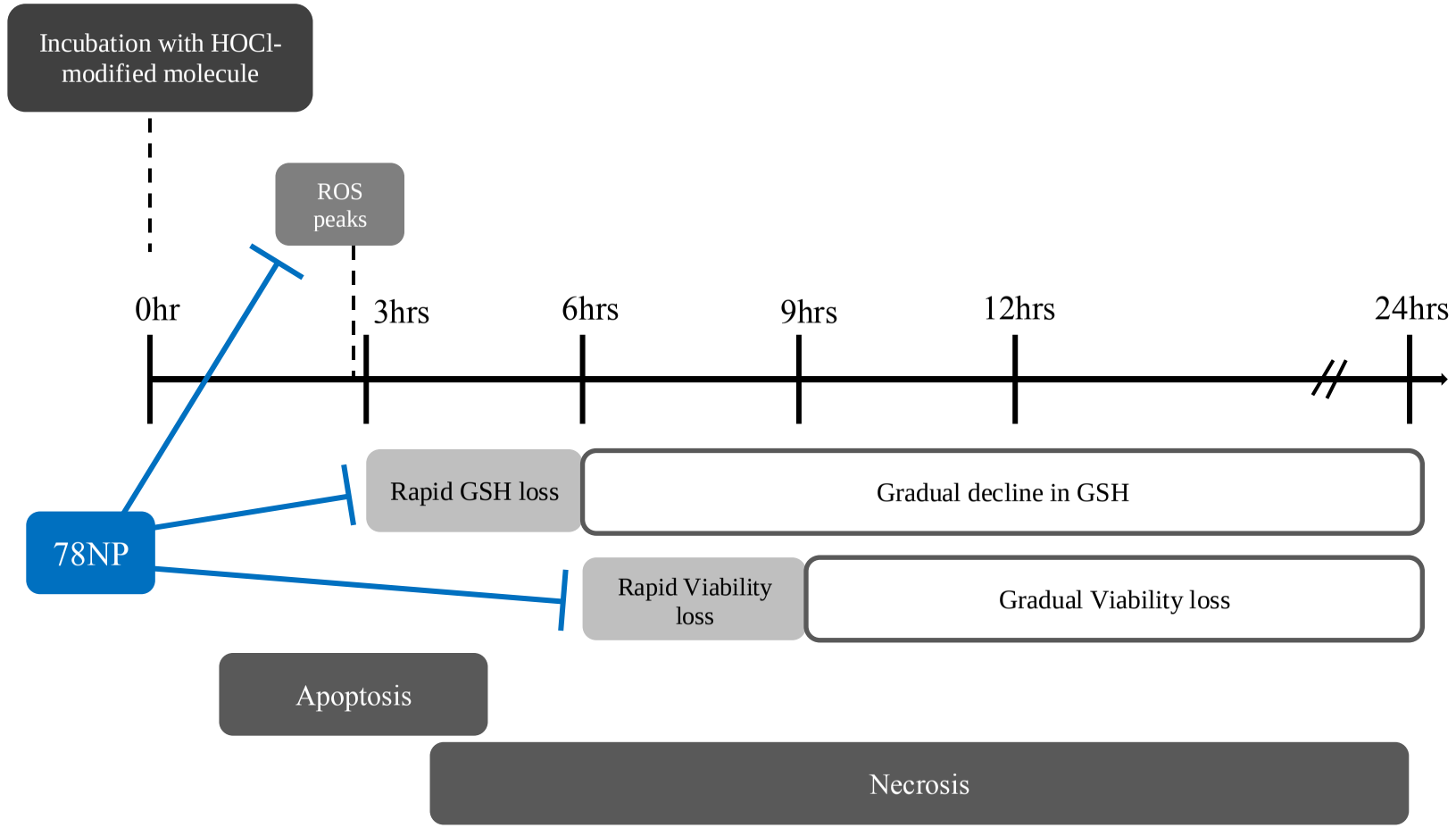


Figure 4.1 Summary of cellular response upon attack with HOCl-modified molecules. Both oxidant types; HOCl-oxLDL and oxALB exhibited similar responses in U937 cells. ROS was detected almost immediately, rising steadily until it peaked at 3 hours. During this time there was a slow and steady viability loss via apoptosis. It appears that ROS levels reached a threshold limit at 3 hours which resulted in a rapid loss of GSH, preceding a marked loss of cell viability though necrotic mechanisms. All these affects could be mitigated by a pre-incubation of 200 µM 78NP before exposure to the oxidants.

Protein oxidation products may act as a novel class of pro-inflammatory mediators, which accumulate and increase oxidative stress and inflammation at atherosclerotic sites. As a result of increased stress, further AOPP formation may occur though the stimulation of leukocytes to produce more oxidants. This positive feedback loop could amplify or maintain oxidative stress and inflammation, thus contribute to atherogenesis. They may also cause cellular death within the necrotic core by inducing intracellular oxidative stress, leading to the depletion of antioxidants and destabilisation of the cell homeostasis for controlling apoptotic cell death, leading to necrosis and growth of the necrotic core.

Mechanisms by which protein oxidation products accelerate atherosclerosis remain to be investigated. Further studies should focus on the toxic agent within the protein moiety of the LDL molecule, and that on AOPPs. This may include: isolation of protein fragments from gels after oxidative modification which could then be tested for toxicity and identified using mass spectrometry; cleavage of selected amino acids off LDL and albumin, with subsequent oxidation to see if toxicity is reduced; and identification of chloramines and reactive aldehydes produced using HPLC. In addition the toxic mechanism of HOCl-oxLDL and oxALB should be further elucidated. What causes the induction of oxidative stress? How is NOX involved, could inhibitors of NOX prevent toxicity of these HOCl-oxidised proteins? Identification of the toxic protein modifications causing these effects would allow for the development of new diagnostic assays, and elucidation of the cytotoxic mechanism may allow for advancement of therapeutic applications.

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